

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

APPLICANTS: Cauwenberghs *et al.* CONFIRMATION NO.: 7478  
APPLICATION NO.: 10/019,740 GROUP NO.: 1641  
FILING DATE: May 8, 2002 EXAMINER: Jung, Unsu

TITLE: Detection of von-Willebrand Factor (VWF) Activity

Mail Stop Amendment  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

**DECLARATION OF DR. JOZEF ARNOUT UNDER 37 C.F.R. § 1.132**

Dear Sir:

I, Dr. Jozef Arnout, hereby declare and state as follows:

1. I am currently the Director of Management of the Biomedical Sciences Group of the University of Leuven in Belgium, supervisor of the hemostasis laboratory of the University Hospitals Leuven, and a professor at the Faculty of Medicine at the University of Leuven. I hold a Masters degree in bioengineering and a Ph.D. in medical sciences, both from the University of Leuven. I have been a member of the editorial boards of the journals Thrombosis and Haemostasis and The Hematology Journal and am a past executive officer of the European Thrombosis Research Organization. I am also a technical advisor to Biokit, S.A. My *curriculum vitae*, which includes my educational and employment history and a list of my publications and patents, is attached hereto as Exhibit A.
2. I am familiar with the subject matter of U.S. Patent Application No. 10/019,740, ("the present application") including the claims as presently on file. I understand that the earliest effective filing date of the present application is July 5, 1999.
3. I have been involved in research related to and laboratory diagnosis of hemostatic disorders, such as von Willebrand's disease, since 1985. I am aware that an assay known

as the von Willebrand factor ristocetin cofactor activity assay (VWF:RCo assay) was developed as early as the early 1970s. Traditionally, the VWF:RCo assay employs exogenous whole platelets, *i.e.*, from a source other than the patient's plasma being analyzed for defects in von Willebrand factor (VWF). The exogenous whole platelets agglutinate in the presence of ristocetin when von Willebrand factor is present in a patient plasma sample.

4. One difference between Applicants' claimed invention and prior art VWF:RCo assays is that Applicants' assay does not use whole platelets. Rather, Applicants' assay uses a soluble form or a portion of glycoprotein 1b( $\alpha$ ) presented by an anti-GP1b( $\alpha$ ) antibody to detect VWF ristocetin cofactor activity of a patient sample. Applicants were the first to use an antibody presented soluble form or a portion of glycoprotein 1b( $\alpha$ ) that is not associated with a platelet to detect the ristocetin cofactor activity of VWF.
5. The VWF:RCo assays known in the prior art have several significant limitations. These limitations have been acknowledged by those of skill in the art and are summarized by Dr. Emmanuel Favaloro in the journal *Seminars in Thrombosis & Hemostasis* (33(4):727-744, (2007), ("Favaloro II", attached as "Exhibit B")) as follows:

"This assay...[was] first described in the very early 1970s....[O]ver the subsequent 35 years or so, several significant limitations to VWF:RCo [(ristocetin cofactor activity assay)] testing have emerged...The classically performed test is *very laborious* and the resultant test values are *highly variable*...For our laboratory to obtain a 'half-decent' result, we have to test patient samples at three plasma dilutions and often have to repeat patient tests; even then, test replicates are still typically *widely disparate*. Each individual test reading takes approximately 5 minutes, and the complete assay on a batch of test samples will typically take the laboratory technician almost an entire day to perform and result. The simple hard truth about the classic VWF:RCo assay is that it would give one an accurate result only after one had (i) tested the patient sample at several dilutions, (ii) retested the patient sample again, and (iii) retested the patient sample using another fresh sample collected on a separate occasion. It is a simple case of averaging as many test results as are available; the more test results averaged, the more reliable the eventual test result obtained. This poor intraassay reproducibility

is compounded by high interassay variability and high interlaboratory variability.

The other main problem with the classic VWF:RCo assay is the assay's lower limit of detection, which typically lies around 10 to 20% VWF [(von Willebrand Factor)]. That is, the classic VWF:RCo assay cannot reliably provide an estimate of VWF below around 20% VWF. This is a serious limitation for an assay that is used to help characterize functional VWF discordance, and given that the vast majority of severe VWD [(von Willebrand disease)] subtypes have levels of VWF below 20%."

The inter-assay variability of [the] VWF:RCo assay...[is] 20 to 40% (Favaloro II at 729-730, emphasis added, attached as "Exhibit B").

6. Further, as taught by Kitchen *et al.* ((2006), Sem. Thromb. Haemost., 32:492-498, "Exhibit C"), inter-laboratory variation for prior art VWF:RCo assays is extremely high, for example, averaging in the range of 40-50% coefficients of variation or higher (Kitchen, abstract, pg. 497, RH col., 1<sup>st</sup> para.). The levels of inter-laboratory variation reported by Kitchen for the VWF:RCo assays assessed by Kitchen are extremely high, well beyond the generally accepted industry standard of less than 15% CV, indicating that these assays cannot provide accurate and reliable measurements of vWF, especially at low levels of vWF, *i.e.*, below 10-20% vWF.
7. Favaloro and Kitchen, as referenced in paragraphs 5 and 6 above, have accurately characterized the known deficiencies of the classical ristocetin cofactor activity assay as understood by skilled artisans in the field of von Willebrand testing and diagnosis. In particular, the prior art ristocetin cofactor activity assay suffers from poor inter-assay variability, poor intra-assay variability, poor reproducibility, and low sensitivity. I have been aware of these deficiencies of the prior art ristocetin cofactor assay discussed above since 1985. It is my understanding that others of skill in the art were aware of these deficiencies in the early 1970s when the prior art VWF:RCo assays were first developed.
8. In particular, the prior art VWF:RCo assays suffer from high inter-assay and intra-assay variability which is represented by high coefficients of variation (%CV), which for prior art VWF assays run as high as 20-40% as reported by Favaloro and 40-50% as reported by Kitchen. High coefficients of variation create a high potential error rate in terms of

false positive or false negative identification of von Willebrand's disease (VWD) (see Favaloro II, p. 730, RH col., 1<sup>st</sup> para.). Because the coefficients of variation (%CV) for prior art VWF:RCo assays are so high, these assays are not precise enough to give accurate measurements at low levels of VWF, as the generally accepted industry standard for variation for these types of assays is less than 15% CV. Accordingly, such assays cannot provide an accurate basis for diagnosis of VWD as such coefficients of variation would significantly and negatively effect the accuracy of a diagnosis of VWD.

9. Furthermore, the prior art VWF:RCo assays are not sensitive enough to reliably and accurately detect the extremely low levels of VWF activity that characterize many VWD patients, in particular, levels below 10-20% VWF (see Favaloro II, p. 730, LH col. 1<sup>st</sup> para.). Given that many VWD patients have VWF levels lower than 20% VWF, even lower than 10%, 5%, or even 1%, for example, the inability of the prior art ristocetin cofactor assays to reliably detect extremely low levels of VWF activity makes it particularly difficult to accurately diagnose these patients.
10. Poor intra-assay and inter-assay variability refers to high coefficients of variation (%CV), while low sensitivity indicates that these prior art VWF:RCo assays are not sensitive enough to accurately and reliably detect low levels of VWF activity, *i.e.*, below 10-20% VWF. The sensitivity of an assay is measured by its limit of quantitation (LOQ), sometimes referred to as functional sensitivity. According to the Clinical and Laboratory Standards Institute (CLSI), the limit of quantitation is defined as the lowest amount of analyte that can be measured with acceptable precision and accuracy under stated experimental conditions. Typically, for diagnostic assays, such as the VWF:RCo, the generally accepted industry standard for precision is 15% CV with accuracy smaller than a stated absolute value or a stated relative value. The problem that has persisted with the prior art VWF:RCo since its earliest development is its inability to accurately detect low levels of VWF (% VWF), *i.e.*, its LOQ is too high. In other words, the prior art VWF:RCo assay is unable to accurately and precisely measure low amounts of VWF, particularly levels less than 10-20% VWF, resulting in a significant, negative impact on the accuracy of a diagnosis of VWF using the prior art VWF:RCo assay. In order to

reliably detect a low level of VWF, an assay must not only be able to detect low levels of VWF, but must do so with low coefficients of variation.

11. Since the 1970s when the prior art VWF:RCo assay was first developed, there has been a persistent need for a VWF:RCo assay that addresses the problems with the prior art VWF:RCo assay discussed in paragraphs 5-10 above, specifically, for an improved ristocetin cofactor activity assay that significantly reduces or eliminates intra-assay and inter-assay variability and improves sensitivity for measuring von Willebrand factor, *i.e.*, an assay that can reliably measure the low levels of von Willebrand factor characteristic of severe von Willebrand disease subtypes with levels of von Willebrand factor well below 20%, with acceptable coefficients of variation. In particular, a ristocetin cofactor assay with the sensitivity to accurately and precisely detect less than 1% VWF would be required to meet this long felt need as many VWF patients have less than 1% VWF and are often subject to misdiagnosis based on the inaccuracy of the prior art ristocetin cofactor assay.
12. Others have been attempting to improve on the prior art VWF:RCo assay since these deficiencies enumerated in paragraphs 5-10 above were first elucidated. For example, laboratories have used stabilized platelets obtained from suppliers rather than preparing their own platelets in order to reduce the variability caused by each laboratory using platelets from different sources. Further, laboratories have automated the VWF:RCo assay to reduce error in detecting agglutination of platelets, to reduce the time-intensive nature of the assay, and to computerize the complex series of calculations involved in measuring platelet agglutination to reduce human error. However, none of these attempts to improve the prior art ristocetin cofactor assay has been as successful in correcting the deficiencies of the prior art ristocetin cofactor activity assay's high inter-assay and intra-assay variability and limited sensitivity, as Applicants' claimed assay. Until the advent of Applicants' claimed invention, the known problems with the prior art VWF:RCo had not been satisfactorily addressed.
13. However, even automated assays and those using stabilized platelets, have not fully addressed all the drawbacks of the classical ristocetin cofactor activity assay as described

by Favaloro. In particular, Favaloro has acknowledged the same, stating that “[a]utomation of test procedures (using instrumentation) has certainly reduced the VWF:RCo assay’s intra-assay and inter-assay variability[,] *but has not alleviated the issue of low-level assay sensitivity*, nor does automation seem to protect laboratories against VWD-identification errors” (Favaloro II, pg. 730, LH col., emphasis added).

14. For example, the product specification for the exemplary commercially available assay (attached as “Exhibit D”) discussed by Dr. Bruguera in his August 15, 2008, declaration, both of which I have reviewed, states the coefficients of variation for the assay, but provides no indication that this VWF:RCo assay can reliably detect levels of VWF less than 20%. In fact, the product specification does not provide the lower limit of quantitation for this assay. However, based on the coefficients of variation for the pathological controls, which typically contain a level of VWF found in a VWD patient, and which are indicated to range from 6.1%-16.2% (within run CV) and from 7.6-16.9% (total CV), it would not be possible to accurately and precisely quantitate low levels of VWF, *i.e.*, below 10-20% VWF, because the coefficients of variation for this assay exceed the generally accepted industry standard for precision which is less than 15% CV.
15. It is my opinion that even further improvements in intra-assay and inter-assay variability were still required as of the earliest effective filing date of Applicants’ application in order to improve the accuracy of the prior art VWF:RCo assays, even with the availability of the commercially available VWF:RCo assay discussed in paragraph 14 above. In other words, the prior art VWF:RCo assays available prior to the earliest effective filing date of Applicants’ application claimed invention and even available today have not satisfactorily resolved the serious problems inherent in the prior art VWF:RCo assays.
16. I have reviewed the data presented at Table 1 in Dr. Bruguera’s August 15, 2008, declaration and also at Table 1 in Dr. Bruguera’s May 18, 2009, declaration. These data indicate that the intra-assay and inter-assay coefficients of variation for Applicants’ claimed assay are still lower than the coefficients of variation for prior art VWF:RCo assays, including the 20-40% coefficients of variation reported by Favaloro II and the 40-


50% coefficients of variation reported by Kitchen. In fact, the data show that the within run and total coefficients of variation for normal and pathological control plasma, when assayed according to Applicants' claimed method, is still lower than the lowest end of the range of within run and total coefficients of variation for normal control and pathological control stated for the exemplary commercially available VWF:RCo assay discussed by Dr. Bruguera in his August 15, 2008, declaration. Applicants improvements in reducing total and within run variation indicates that the mean % VWF activity detected across samples using Applicants' claimed invention is more accurate, and as a result, a more accurate diagnosis of VWD can be made. Accordingly, Applicants' lower coefficients of variation over the prior art VWF:RCo assays, including the exemplary commercially available VWF:RCo assay discussed by Dr. Bruguera in his August 15, 2008, declaration, demonstrate an extremely significant and valuable improvement in reducing the inter-assay and intra-assay variability over the prior art VWF:RCo assay.

17. Further, Applicants' assay has a lower limit of 0.27% VWF with a coefficient of variation (CV) of 7.9% at that lower limit, meeting the generally accepted industry standards for precision and accuracy. Accordingly, unlike the prior art VWF:RCo assays which have coefficients of variation that are too large to precisely measure low levels of VWF, Applicants' assay can precisely and accurately detect levels of VWF as low as 0.27% with acceptable coefficients of variation. Applicants' assay meets the long felt need in the art for a VWF:RCo assay with improved sensitivity to low levels of VWF because it has an LOQ of 0.27% which is lower than any VWF:RCo assay available prior to the earliest effective filing date of this application. This level of sensitivity is at least 30-60 times lower than the 10-20% VWF lower level of sensitivity limit of the prior art VWF:RCo reported by Favaloro (Favaloro II, p.730, LH col., 1<sup>st</sup> para.) and is an extremely significant and valuable improvement over prior art VWF:RCo assays. Prior to Applicants' invention, it was not possible to accurately and reliably detect such low levels of vWF using a prior art VWF:RCo assay.
18. The improvements achieved by Applicants' claimed invention with respect to the improved low level of sensitivity and reduced inter-assay and intra-assay variation are

attributable to the use of a soluble form or portion of glycoprotein 1b( $\alpha$ ) (GP1b $\alpha$ ) presented by an anti-GP1b $\alpha$  antibody to detect the VWF ristocetin cofactor activity.

19. As of the earliest effective filing date, Applicants assay was the only known VWF:RCO assay with the sensitivity to accurately and precisely detect less than 1% VWF with acceptable levels of inter- and intra-assay variability (CV%). Because Applicants' claimed invention has the ability to reliably and accurately detect levels of VWF well below 1%, *i.e.*, as low as 0.27%, and because it significantly reduces levels of intra-assay and inter-assay variability, the long felt need in the art for an improved assay which has persisted in the art since the 1970s is met by Applicants' claimed invention.
20. I further declare that all statements made in this Declaration are of my own knowledge, are true, and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 2003-05-21

By   
Dr. Jozef Arnout



**Jozef ARNOUT**

Curriculum vitae, May 2009

Personal Data

Office: Management Biomedical Sciences  
University of Leuven (KU Leuven)  
Campus Gasthuisberg O&N2, bus 700  
Herestraat 49  
B-3000 Leuven, Belgium  
(Tel 016/330284, Telefax 016/330212)  
e-mail: jef.arnout@gbiomed.kuleuven.ac.be)

Research affiliation Center for Molecular and Vascular Biology  
Department of Molecular and Cellular Medicine  
Biomedical Sciences Group  
University of Leuven  
Campus Gasthuisberg O&N1, bus 911  
Herestraat 49  
B-3000 Leuven, Belgium  
(Tel 016/345775, Telefax 016/345990;

Education

1977: Bio-ingeneer, University of Leuven

1981: Certificate "Geaggregeerde voor het HSO"

1994: Doctor in Medical Sciences, KU Leuven

Academic Appointments within the University of Leuven

1978-1980: Research Assistant at the Laboratory for Applied Carbohydrate Chemistry,  
University of Leuven

1980-1981: Research Assistant at the Laboratory for Applied Organic Chemistry,  
University of Leuven

Since 1982: Research Associate at the Center for Molecular and Vascular Biology,  
University of Leuven (Director: Prof. D. Collen)

1985: Supervisor Hemostasis Laboratory University Hospitals Leuven

1999: Associate professor, Faculty of Medicine, University of Leuven

- 2003: Professor , Faculty of Medicine, University of Leuven
- 2005: Director experimental animal center University of Leuven
- 2006: Director of Management, Biomedical Sciences Group, University of Leuven  
(Faculty of Medicine, Faculty of Pharmaceutical Sciences, Faculty of  
Kinesiology and Rehabilitation Sciences)

#### Awards and Honours

- 1999: Investigators Recognition Award for contributions to Haemostasis and  
Thrombosis (XVIIth ISTH Congress, Washington)
- 1999: Prof. Marc Verstraete Prijs for contributions to Thrombosis and Haemostasis  
1996-1999.

#### Editorial Tasks in Scientific Journals

- 1993: Associate Editor, Thrombosis and Haemostasis
- 1999-2007 Member of the Editorial Board, Thrombosis and Haemostasis
- 1999: Member of the Editorial Board, The Hematology Journal

#### Membership in Scientific Organizations

- 1987: Secretary Scientific Committee of the XIth Congress of the International  
Society on Thrombosis and Haemostasis
- 1993: Member of the International Society for Thrombosis and Haemostasis
- 1993: Member of the Expert Committee for the Evaluation of Quality in  
Hemostasis Testing
- 1994: Vice chairman of the sixth International Symposium on Antiphospholipid  
Antibodies, Leuven, 1994
- 1994: Secretary General at the XIIth International Congress on Fibrinolysis, 1994
- 1994-2000: Elected member of the Scientific and Standardization Committee (SSC) of  
the ISFT
- 1997: Advisor of the ECAT Foundation
- 1999-2003 Chairman of the SSC Scientific Subcommittee on Lupus Anticoagulant/  
Phospholipid-dependent Antibodies

2005-2008      Executive officer European Thrombosis Research Organisation

Research Areas

- pathophysiology of the haemostasis and thrombosis
- antiphospholipid syndrome
- platelet physiology and pharmacology

Publications

The author's scientific output consists of more than 125 research papers in peer-reviewed international journals and 10 survey articles in books.

2007-01-03

Jozef ARNOUT

**I. Publications in international peer reviewed journals**

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2. Gresele P., Zoja C., Deckmyn H., Arnout J., Vermynen J., Verstraete M. Dipyridamole inhibits platelet aggregation in whole blood. *Thrombosis and Haemostasis* 50, 852-856, 1983.
3. Gresele P., Deckmyn H., Arnout J., Lemmens J., Janssens W., Vermynen J. BM 13.177, a selective blocker of platelet and vessel wall thromboxane receptors, is active in man. *Lancet* i, 991-994, 1984.
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5. Deckmyn H., Gresele P., Arnout J., Todisco A., Vermynen J. Prolonging prostacyclin production by nafazatrom and dipyridamole. *Lancet* ii, 410-411, 1984.
6. Essien E.M., Arnout J., Deckmyn H., Vermynen J., Verstraete M. Blood changes and enhanced thromboxane and 6-keto prostaglandin F<sub>1a</sub> production in experimental acute plasmodium bergeri infection in hamsters. *Thrombosis and Haemostasis* 51, 362-365, 1984.
7. Gresele P., Deckmyn H., Arnout J., Zoja C., Vermynen J. Lack of synergism between dazoxiben and dipyridamole following administration to man. *Thrombosis Research* 37, 231-236, 1985.
8. Vanrenterghem Y., Roels L., Lerut T., Gruwez J., Michiels P., Gresele P., Deckmyn H., Colucci M., Arnout J., Vermynen J. Thrombo-embolic complications and haemostatic changes in cyclosporin-treated cadaveric kidney allograft recipients. *Lancet* i, 999-1002, 1985.
9. Vermynen J., Deckmyn H., Arnout J., Gresele P. A. Schmidt Memorial Lecture : Peroxides in haemostasis and thrombosis. *Haemostasis* 15, 8-9, 1985.
10. Gresele P., Arnout J., Deckmyn H., Vermynen J. Combining antiplatelet agents : potentiation between aspirin and dipyridamole. *Lancet* i, 937-938, 1985.
11. Arnout J., Van Russelt M., Deckmyn H., Vermynen J., Fiocchi R., Lijnen P., Amery A. Platelet hypersensitivity to serotonin after prolonged ketanserin intake ? *J. Cardiovasc. Pharmacol.* 7 (Suppl. 7), S20-S22, 1985.

12. Deckmyn H., Zoja C., Arnout J., Todisco A., D'Hondt L., Vanden Bulcke F., Hendrickx N., Gresele P., Vermynen J. Partial isolation and function of the prostacyclin regulating plasma factor. *Clin. Science* 69, 383-393, 1985.
13. Gresele P., Bounameaux H., Arnout J., Perez-Requejo J.L., Deckmyn H., Vermynen J. Thromboxane A2 and prostacyclin do not modulate the systemic hemodynamic response to cold in humans. *J. Lab. Clin. Med.* 106, 534-541, 1985.
14. De Maeyer P., Deckmyn H., Arnout J., Baert A.L., Vermynen J. Intravenous ionic contrast media cause local prostacyclin release in man. *Investigative Radiology* 20, 871-873, 1985.
15. Gresele P., Arnout J., Deckmyn H., Vermynen J. Mechanism of the antiplatelet action of dipyridamole in whole blood : modulation of adenosine concentration and activity. *Thrombosis and Haemostasis* 55, 12-18, 1986.
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21. Gresele P., Arnout J., Deckmyn H., Huybrechts E., Pieters G., Vermynen J. Role of proaggregatory and antiaggregatory prostaglandins in hemostasis. Studies with combined thromboxane synthase inhibition and thromboxane receptor antagonism. *J. Clin. Invest.* 80, 1435-1445, 1987.
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- human platelet activation in vitro and in vivo: a comparison with aspirin. *Blood* 75, 646-653, 1990.
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# An Update on the von Willebrand Factor Collagen Binding Assay: 21 Years of Age and Beyond Adolescence but Not Yet a Mature Adult

Emmanuel J. Favaloro, Ph.D., M.A.I.M.S.<sup>1</sup>

## ABSTRACT

von Willebrand disease (VWD) is considered to be the most common inherited bleeding disorder. It is diagnosed after a clinical and physical review, with personal and familial evidence of (primarily mucocutaneous) bleeding, and confirmed by laboratory testing. The latter typically entails initial plasma testing of factor VIII coagulant (FVIII:C), von Willebrand factor (VWF) protein (antigen; VWF:Ag), and VWF function, which has classically been assessed using the ristocetin cofactor (VWF:RCo) assay. More recent attention has focused on another functional VWF assay, the collagen binding (VWF:CB) assay, as a possible replacement for the VWF:RCo assay or as a supplementary test of VWF adhesive "activity." Additional laboratory testing can comprise a battery of confirmatory and VWD subtype assisting assays, including assessment of VWF:multimers. This review updates our knowledge of VWD diagnostics with a particular emphasis on the VWF:CB assay. There is good evidence now in place that an optimized VWF:CB assay can significantly reduce the diagnostic error rate otherwise arising from the use of a test panel restricted to including the VWF:RCo assay as the sole functional VWF assay. Nevertheless, the VWF:CB assay should not be used to wholly replace the VWF:RCo assay in phenotypic testing but rather as a supplementary assay. However, with some thought and justification, the VWF:CB assay can be used to partly replace the VWF:RCo assay in some "screening" applications and can also be used to abrogate the need to perform routine VWF:multimers in most test cases.

**KEYWORDS:** von Willebrand Factor, VWF, von Willebrand disorder, von Willebrand disease, VWD, collagen binding, VWF:CB, ristocetin cofactor, VWF:RCo, multimers

Several events have prompted a review of the current status of diagnostics for von Willebrand disease (VWD) and subsequently stimulated the preparation of this article. First and foremost was the recognition that

the first published description of this assay we now call the von Willebrand factor (VWF) collagen binding (VWF:CB) assay was 21 years ago, in 1986, by Brown and Bosak.<sup>1</sup> The VWF:CB assay was then coevaluated

<sup>1</sup>Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, Westmead, Australia.  
Address for correspondence and reprint requests: Dr. Emmanuel J. Favaloro, Ph.D., M.A.I.M.S., Department of Haematology, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, SWAHS, Westmead, NSW, 2145, Australia (e-mail: emmanuel.favaloro@swahs.health.nsw.gov.au).

Hot Topics I: A Potpourri of Current Issues and Controversies in Thrombosis and Hemostasis; Guest Editor, Emmanuel J. Favaloro, Ph.D., M.A.I.M.S.

Semin Thromb Hemost 2007;33:727-744. Copyright © 2007 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662.  
DOI 10.1055/s-2007-1000364. ISSN 0094-6176.

with the ristocetin cofactor (VWF:RCo) assay regarding its ability both to detect VWD and to identify and discriminate potential type 2 VWD subtypes. Although this study was a relative landmark, being the first description of the potential utility of the VWF:CB assay, the data reported and the conclusions raised did little in itself to endorse the continued use of the VWF:CB assay in VWD diagnostics. The study comprised a very small group of VWD patients and normal individuals and used a single source of collagen to coat enzyme-linked immunosorbent assay (ELISA) plates. The comparative data between the VWF:CB and VWF:RCo assays was not strikingly dissimilar, and in hindsight, the VWF:CB assay used was not really optimized for discrimination of high-molecular-weight (HMW) VWF. Indeed, the VWF:CB story might well have ended there, except that several other workers also decided to test the utility of this novel assay. I was one of those workers and was sorely tempted at that time by the perception that the VWF:CB assay could potentially replace the need to perform the laborious and highly variable VWF:RCo assay.

The second event that prompted this article was not actually a single event but rather comprises a composite of events—the ongoing communications that I receive, primarily by e-mail these days, expressing confusion regarding the VWF:CB assay itself, as well as its place within the diagnostics and therapy of VWD. This confusion is somewhat bemusing. On the one hand, one might expect that the situation should be reasonably clear, particularly given the amount of literature on the VWF:CB assay over the past 15 years. On the other hand, several developments continue to cloud the situation. Many queries still relate to issues of assay standardization and to which collagens we should be using for this assay. Should we be using purified type I, purified type III, or type I/III collagen mixtures? Should these be of equine, bovine, or human origin? Should these originate from skin, tendon, or placenta? More recently, human collagen type VI has also emerged as a possible contender. I am happy to repeat my current stance, which has not changed since around the year 2000—several studies have been published over the years, and all *comparative* studies to date have shown that VWF:CB assays based on type I/III collagen mixtures are best at distinguishing HMW forms of VWF, and hence therefore generally better at discriminating plasma samples from type 2A and 2B VWD from those from type 1 VWD and normal individuals.<sup>2–5</sup> It is true that *noncomparative* studies have shown that VWF:CB assays based on purified type III collagen are capable of discriminating HMW VWF and differentiating type 2 VWD to some degree<sup>4,6</sup>; however, the *comparative* studies show that discrimination is better with the type I/III collagen mixture-based assays.

Nevertheless, most commercial suppliers continue to manufacture VWF:CB assay kits based on purified type III collagen preparations. Although I think I can explain the reasoning behind this, the potentially negative downstream effect is that most clinical studies that need to utilize a commercial kit VWF:CB procedure are therefore “forced” into using assays based on purified type III collagen, and these might represent nonoptimally HMW-sensitive procedures and produce suboptimal results, should the study be intending to use assay results as surrogate markers for HMW VWF.

Most of the other communication queries I referred to previously relate to the relative utility of the VWF:CB assay over that of the VWF:RCo assay and to the question of whether or not the VWF:CB assay can now replace the VWF:RCo assay in VWD diagnostics. Although our laboratory originally evaluated the VWF:CB assay with such a view in mind, this did not ultimately occur (except in part), and we still continue to perform VWF:RCo testing for many cases of “?VWD” under investigation. Instead, the VWF:CB assay has, within our laboratory, largely replaced the need to perform VWF:multimer testing on the vast majority of test cases, but more on this and our rationale later.

The third event to prompt this article is again actually a series of events—the plethora of recent studies and publications regarding genetic mutations in VWD and the resulting ability to better identify “true” cases of VWD, enabling the reassessment of these cases in terms of phenotypic test profiles.

The final event that prompted this article was a full-page (presumably paid) advertisement for a commercial VWF:CB assay that recently appeared on the back page of the August 2007 issue of the prominent journal *Thrombosis and Hemostasis*, and which prompted me to ask once again: Has the VWF:CB assay, at “age 21,” finally come of age?

## BACKGROUND I: VWD AND THE ROLE OF LABORATORY TESTING IN ITS DIAGNOSIS

VWD is considered to be the most common inherited bleeding disorder. Although the calculated incidence varies according to the method used, most estimates are around 0.1 to 1% of the general population.<sup>7</sup> VWD is diagnosed after a clinical and physical review, with personal and familial evidence of (primarily mucocutaneous) bleeding, and confirmed by laboratory investigation.<sup>8,9</sup> The latter typically entails initial plasma testing (laboratory “screening”) of factor VIII coagulant (FVIII:C) and VWF protein (antigen; VWF:Ag) and function, with this classically assessed using VWF:RCo assay. More recent attention has focused on the VWF:CB assay as a possible replacement for the VWF:RCo assay or as a supplementary test of VWF adhesive “activity.” Other putative “functional” (VWF “activity”)

assays have also recently emerged.<sup>9,10</sup> Additional laboratory testing can comprise a battery of confirmatory and VWD subtype assisting assays. These may include assessment of VWF:multimers, ristocetin-induced platelet agglutination (RIPA), and/or VWF-factor VIII binding (VWF:FVIIIIB).<sup>8,9</sup>

Most simply defined, VWD is characterized by defective function in, and/or reduced levels of, VWF. There are six defined VWD types, comprising type 1, type 2 (with breakdown into 2A, 2B, 2M, and 2N), and type 3.<sup>8,9</sup> Types 1 and 3 are quantitative defects. Type 1 individuals produce low levels of otherwise functionally normal VWF, and VWF is generally absent in type 3 VWD. In contrast, type 2 VWD represents qualitative defects characterized by the presence of dysfunctional VWF, with the particular defect or dysfunction characterized within the subtype. Although absolute (total) plasma levels of VWF protein in type 2 VWD are also generally low, they might on occasion be normal.

In brief, type 2N VWD is characterized by a defect in the binding of plasma VWF to FVIII, and types 2A, 2B, and 2M present with either a relative absence of HMW VWF and/or some other inherent VWF dysfunction.<sup>8,9</sup> The main defect presenting in type 2A is a loss of HMW and intermediate-molecular-weight (IMW) VWF, and the main defect presenting in type 2B VWD is a heightened affinity of plasma VWF to platelet glycoprotein Ib/IX, leading to consequent loss of (primarily) HMW VWF from the circulation. Type 2M VWD is a qualitative defect that presents with dysfunctional VWF but that is not associated with a loss of HMW VWF. Thus, the defect presenting in type 2M VWD is an inherent functional "qualitative" defect, and the specific defect(s) that present depends on the genetic mutation evident. Type 2M VWD is very difficult for many people to conceptualize; in fact, this may best be imagined as a composite of possible qualitative disorders that otherwise do not fit within the other type 2 VWD categories.<sup>8,9,11,12</sup>

Identification of type 2 VWD or its discrimination from type 1 VWD, and potentially type 3 VWD and even normal individuals, is most simply accomplished by phenotypic laboratory testing and assessment of the relative proportions of functional VWF compared with the total VWF protein present in the same plasma sample<sup>9</sup>; or in other words, by the relative proportion of VWF:RCo or VWF:CB or VWF:FVIIIIB or FVIII:C compared with VWF:Ag, and most typically expressed as a ratio of assay values. Although this is expanded on later, type 1 VWD will present with low and proportionally similar levels of VWF detected by all the VWF assays (i.e., VWF:Ag, VWF:RCo, VWF:CB), and thus will give a test ratio of close to 1.0 irrespective of the assays compared. Type 3 VWD will generally present with an absence of VWF, again irrespective of the assay used; nevertheless, estimation of assay ratios are not

recommended in type 3 VWD because of assay sensitivity issues at low levels of VWF and resultant false or meaningless values generated consequent to very low or zero numerator values. Type 2 VWD will present with low relative FVIII:C, VWF:RCo and/or VWF:CB compared with VWF:Ag and it is the specific pattern of the test discordance in results that can help guide further investigation to pinpoint the specific dysfunctional VWD subtype presenting.

## **BACKGROUND II: STRENGTHS AND LIMITATIONS OF THE CLASSIC TEST PROCEDURES OF VWF:RCO AND VWF:MULTIMER ASSESSMENT**

### **VWF:RCo Assay**

This assay represents the original functional VWF test, being first described in the very early 1970s.<sup>13-15</sup> The introduction of the VWF:RCo test at that time caused a revolution in VWD diagnostics by providing laboratories with their first real tool to enable proper characterization of qualitative dysfunctional VWD cases, something that had eluded them with previous limited testing. Nevertheless, over the subsequent 35 years or so, several significant limitations to VWF:RCo testing have emerged.

First, the classically performed test (using a platelet agglutination procedure in a platelet aggregometer) is very laborious, and the resultant test values are highly variable. As a "hands-on" laboratory scientist, I have some considerable personal experience in this. It can take the laboratory several hours to obtain an estimate of the VWF:RCo level in a given patient (test) plasma, and even then such an estimate could not be considered undeniably accurate. The classic testing procedure is a time-linear assay; because of assay drift, a calibration curve using normal plasma should be performed at the start of the assay and repeated at its end. If the procedure is investigating a large number of patient samples, it is good laboratory practice to repeat the calibration curve several times (e.g., we re-perform this calibration every six patient samples). For our laboratory to obtain a "half-decent" result, we have to test patient samples at three plasma dilutions and often have to repeat patient tests; even then, test replicates are still typically widely disparate. Each individual test reading takes ~5 minutes, and the complete assay on a batch of test samples will typically take the laboratory technician (in my laboratory, it was often me) almost an entire day to perform and result. The simple hard truth about the classic VWF:RCo assay is that it would give one an accurate result only after one had (i) tested the patient sample at several dilutions, (ii) retested the patient sample again, and (iii) retested the patient using another fresh sample collected on a separate occasion. It is a simple case of

averaging as many test results as are available; the more test results averaged, the more reliable the eventual test result obtained. This poor intraassay reproducibility is compounded by high interassay variability and high interlaboratory variability, but more on that later.

The other main problem with the classic VWF:RCo assay is the assay's lower limit of detection, which typically lies around 10 to 20% VWF. That is, the classic VWF:RCo assay cannot reliably provide an estimate of VWF below around 20% VWF. This is a serious limitation for an assay that is used to help characterize functional VWF discordance, and given that the vast majority of *severe* VWD subtypes have levels of VWF below 20%.

What the above issues mean in practice is that using the VWF:RCo assay as the base VWF functional assay, together with the VWF:Ag and FVIII:C assays, to comprise the VWD screening test panel (i.e., to provisionally identify VWD), and to then characterize these cases as either functionally VWF concordant (i.e., types 1 or 3 VWD) or discordant (i.e., type 2 VWD) is not always going to be successful. The extent of the possible error rate in the diagnosis and subclassification of VWD using VWF:RCo as the sole VWF functional assay is only now beginning to emerge—and it is a serious problem, as will be highlighted later. Automation of test procedures (using instrumentation) has certainly reduced the VWF:RCo assay's intraassay and interassay variability but has not alleviated the issue of low-level assay sensitivity, nor does automation seem to protect laboratories against VWD-identification errors.<sup>10,16</sup>

As a rough guide, the interassay variability of test procedures used to identify VWD rises in the order FVIII:C < VWF:Ag < VWF:CB < VWF:RCo, with respective median coefficient of variation (CV) values approximating <10%, 10 to 15%, 15 to 25%, and 20 to 40%. It is interesting that CVs are lowest for FVIII:C, a clot-based assay; this most probably reflects some uniformity of test procedures (most laboratories perform one-stage clotting assays) and reasonable international standardization of plasma calibrant material. VWF:Ag assays have a somewhat higher CV, most likely due to procedural assay variation and reduced between-assay standardization. For example, VWF:Ag assays using latex immunoassay (LIA) procedures tend to provide higher detected VWF values compared with those performed by ELISA.<sup>10</sup> Assay variation in VWF:CB is somewhat higher again; this is in part due to even greater assay procedural variation and poorer international standardization but also reflects the fact that these assays detect a smaller proportion (probably around 30%) of the total VWF plasma pool (i.e., primarily the HMW VWF fraction). An analogy is available in thrombophilia testing, where CVs for free protein S assays tend to be greater than those for total protein S. Free protein S reflects the functional pool

(~40%) of the total protein S available in plasma, and this reduced detection base will accordingly result in greater assay variation; nevertheless, free protein S is considered the more important of the two test parameters to measure.

Despite its poor assay reproducibility, and thus high potential error rate in terms of false positive and false negative identification of VWD, VWF:RCo is still seen as an important assay within VWD diagnostics, and when used appropriately and with full knowledge of these stated limitations, will both help to identify and sub-characterize VWD. It is also an imperative assay for the proper identification and characterization of the most common presentation for Type 2M VWD (see later).

### VWF:Multimer Evaluation

The assessment of VWF:multimers is also an established test procedure within VWD diagnostics but is quickly disappearing in general laboratories. This test involves gel electrophoresis of test plasma and separation of VWF forms according to molecular size. Thus, the test has some utility in terms of its ability to identify qualitative defects in VWD, primarily those in which the HMW forms are missing (e.g., types 2A and 2B VWD) or in which other qualitative abnormalities may be evident (e.g., abnormal VWD banding or triplet structures). However, the test is very laborious and technically demanding, even much more so than VWF:RCo. A laboratory has to invest a lot of time and effort into obtaining good multimer results, and very few laboratories these days are doing so. As an example, of ~40 laboratories within Australia that perform some form of phenotypic testing for VWD, only two perform VWF:multimers. If we grade the VWF assays for time and complexity, VWF:Ag would grade as one "star," the VWF:CB about two "stars," the VWF:RCo about three "stars," and the VWF:multimer assay about five "stars." Put another way, a laboratory scientist could feasibly test some 60 individual test plasmas for VWF:Ag, VWF:CB, and FVIII:C on a single day and obtain decent quantitative data for most cases. One would be hard pressed to test even 20 individual plasmas for VWF:RCo using the classic agglutination procedure on a single day, and it would typically take around 3 to 4 days to obtain results for around 14 samples for VWF:multimers. There is also some misconception among clinicians and hematologists that VWF:multimer patterns do not lie. In truth, cross-laboratory evaluation using VWF:multimer assessment shows considerable variance in quality, and interpretation, so that it is entirely feasible to get false interpretations of type 1 VWD when testing type 2 VWD plasma and false interpretations of type 2 VWD when testing type 1 VWD plasma.

### **LIMITATIONS OF THE CLASSIC TEST PANELS COMPRISING VWF:RCO AND VWF:MULTIMER ASSESSMENT IN THE IDENTIFICATION OF VWD AND THE DISCRIMINATION OF VWD SUBTYPES**

It has been mentioned that the VWF:RCo assay is laborious and highly variable, and that multimer testing is even more laborious and should not be viewed as foolproof. Although the VWF:CB assay should also not be viewed as foolproof, a properly optimized VWF:CB assay is less laborious than either the VWF:RCo assay or multimer testing, and is also generally less variable than the VWF:RCo assay. If the laboratory is already performing VWF:Ag by ELISA, it is a simple progression to also undertake VWF:CB by ELISA. So, the question of whether the VWF:CB assay can be used to replace at least some of the testing performed by VWF:RCo assay and multimer analysis often arises. In short, for reasons highlighted in this review, our laboratory's current view is that the VWF:CB assay cannot entirely replace either VWF:RCo assay or multimer testing, but with the right strategies in place, it can be used to reduce the reliance on these more laborious and/or variable assay systems and that this will also lead to reduction of errors in diagnostics and improvement of both the detection of VWD and the identification of qualitative (i.e., type 2) VWD subtypes.

Before detailing our laboratory's diagnostic strategy, some additional background information may be helpful. We have consistently reported in cross-laboratory studies that laboratories using a core plasma testing set of FVIII:C, VWF:Ag, and VWF:RCo will correctly identify a type 2 (A or B) VWD as such in only around 75% of test cases.<sup>10,16</sup> That is, in around 25% of such test cases, a laboratory will misidentify a type 2 (A or B) VWD test plasma as either being normal or type 1 VWD, and this misidentification error rate will reduce to around 10% in laboratories that use a VWF:CB assay. Significant reductions in misidentifications can also be shown for cases of testing of type 1 VWD (misidentified as type 2) or testing of normal samples (misidentified as type 2) after incorporation of the VWF:CB assay into the VWF test panel.

Although this finding has now been replicated and reported several times by our organization, the question regarding how often VWD misidentifications might actually be occurring in broader clinical practice is only recently coming to light. Several VWD genetic studies have now been reported, and these have determined some interesting genotypic versus phenotypic versus clinical bleeding severity correlations.<sup>17-21</sup> Amid the data, the following has also emerged. In a recently published European-based study involving 14 VWD treatment centers in nine European countries, of 150 index cases originally identified as type 1 VWD, a subgroup of 57 patients were later identified to have

abnormal multimer patterns, and these showed a high prevalence of VWF gene mutations (94%, or 54 of 57 cases).<sup>18</sup> In contrast, fewer mutations could be identified (55%, or 51 of 93 cases) in those cases with qualitatively normal VWF. This study concluded that "About one third of the Type 1 VWD cases recruited could be reconsidered as Type 2. The remaining group could be considered "true" Type 1 VWD, although mutations were found in only 55%."<sup>18</sup> Although not specifically stated, it is likely that the majority of these initial type 1 VWD misidentifications comprised laboratory assessments using assay panels comprising VWF:Ag and VWF:RCo without VWF:CB (as this assay is simply less often performed by European laboratories). An important fact to remember here is that these were VWD treatment centers; so the likelihood of false identification of type 2 VWD individuals as type 1 VWD can be deduced to be even higher for nonexpert VWD testing laboratories.

Recent studies from two other groups would have one conclude in a similar vein.<sup>19-21</sup> Thus, of a total of 194 families submitted to the Canadian type 1 VWD study, referred from 13 tertiary care, academic health centers across Canada, 12 families were reclassified as type 2 VWD after reevaluation of the hemostasis studies from both the referral clinic and the central laboratory.<sup>19</sup> In a subsequent analysis, a further 10 index cases were found to have either loss of HMW VWF or abnormal triplet patterns using multimer analysis, and a further 11 cases with normal multimers were found to have low VWF:RCo/VWF:Ag (RCo/Ag) ratios (<0.6).<sup>20</sup> In total, then, some 33 of 194 (17%) index cases originally identified as type 1 VWD might be reclassified as type 2 VWD after this study's reevaluation. Again, one can conclude that the majority of these initial type 1 VWD misidentifications comprised laboratory assessments using assay panels containing VWF:Ag and VWF:RCo without VWF:CB (as this assay is simply not performed by the majority of laboratories in Canada). Another study from the UK Haemophilia Centre Doctors' Organization (UKHCDO) involved 40 families recruited through the national network of Comprehensive Care Haemophilia Centres affiliated with UKHCDO and diagnosed to have type 1 VWD.<sup>21</sup> A family could be included in this study if "a consultant hematologist at a hemophilia center had made a diagnosis of type 1 VWD [according to previous UKHCDO guidelines<sup>22</sup>], there were recorded VWF ristocetin cofactor activity (VWF:RCo) levels of below 50 U/dL, the ratio of VWF:RCo to VWF antigen (VWF:Ag) was greater than 0.7, the plasma VWF multimer profile was normal, individuals had a history of significant mucocutaneous bleeding, and there was more than one family member with a diagnosis of type 1 VWD."<sup>21</sup> After a review of submitted cases by the authors, six (13.6%) families were rediagnosed as having type 2 VWD.<sup>21</sup>

# **CLARIFYING THE ROLE OF THE VWF:CB ASSAY AS PART OF A PANEL OF LABORATORY TESTS TO ASSIST IN THE IDENTIFICATION OF VWD AND THE DISCRIMINATION OF VWD SUBTYPES**

It has been specifically noted that diagnostic errors are reduced significantly by incorporation of the VWF:CB assay.<sup>10,16</sup> The following provides a summary of how the VWF:CB might be incorporated into the diagnostic test process to facilitate better identification and classification of VWD.

## **Normal Plasma and Type 1 VWD**

Normal plasma contains a normal level of VWF; moreover, the level of VWF detected is similar (or "concordant") irrespective of the VWF assay employed (i.e., VWF:Ag, VWF:CB, or VWF:RCO). Accordingly, the ratio of RCo/Ag and VWF:CB/VWF:Ag (CB/Ag) is going to be close to 1.0 (in practice, it is generally > 0.7).<sup>9</sup> Type 1 VWD presents with a low level of VWF; however, because this VWF is qualitatively normal, the level of VWF detected is again similar irrespective of the assay used (i.e., concordant-like normal plasma, RCo/Ag, and CB/Ag ratios are still going to be around 1.0 [or > 0.7]; see Table 1).

## **Type 3 VWD**

Although some forms of type 3 VWD may present with low detectable levels of VWF, type 3 VWD is generally characterized by an absence of plasma VWF (irrespective of the VWF assay used). Thus, the level of VWF detected using VWF:Ag, VWF:RCO, and VWF:CB should be similar (i.e., concordant) and around 0. Nevertheless, there are several reasons why we do not recommend attempts to determine assay ratios for levels of VWF under 10% or when type 3 VWD is suspected. Most importantly, low-level detection assay sensitivity issues are such that VWF is sometimes falsely identified

in these patients upon testing. This is a bigger problem with VWF:RCO than with VWF:Ag and VWF:CB, but even in these assays, levels of VWF up to 10% might be falsely identified.<sup>9,23,24</sup> This will lead to spurious and false assay ratio values (e.g., VWF:Ag of 3% and VWF:CB of 1% will give a CB/Ag ratio of 0.33, which might lead to an incorrect "conclusion" of type 2 functional VWF discordance).

## **Type 2 VWD**

In contrast with types 1 and 3 VWD, which are quantitative VWF defects, type 2 VWD is characterized by "qualitative" defects, or by the presence of dysfunctional VWF, with the particular defect characterized within the subtype.<sup>8,9</sup>

## **TYPE 2N VWD**

This is characterized by a defect in the binding of plasma VWF to FVIII, which leads to a low relative plasma FVIII:C to VWF. However, laboratory testing realities and genetic variation mean that this is an inconsistent finding. In particular, some laboratory FVIII:C assays may provide "false" high values for FVIII:C, thus yielding fairly normal FVIII/VWF ratios and therefore masking the presence of type 2N VWD. Accordingly, a normal ratio of FVIII/VWF does not exclude type 2N VWD, and VWF:FVIII:B assays should always be performed to identify or exclude type 2N VWD. A low VWF:FVIII:B/VWF:Ag ratio (generally < 0.6 or < 0.7) is characteristic of type 2N VWD.

## **TYPES 2A, 2B, AND 2M VWD**

These present with either a relative absence of HMW VWF and/or some inherent VWF dysfunction, and these are therefore generally characterized by low RCo/Ag and/or CB/Ag ratios. In brief, the main defect presenting in type 2A is a loss of HMW and IMW VWF; hence, type 2A VWD will typically present with very low RCo/Ag and very low CB/Ag ratios. The main

**Table 1 Expected Laboratory Phenotypic VWF Assay Findings in VWD\***

Assay Parameter	VWD Subtype					
	1	2A	2B	2M <sup>†</sup>	2N	3
VWF:Ag	Low (< 50%)	Low/normal	Low/normal	Low/normal	Low/normal	Absent
VWF:RCO	Low (< 50%)	Low	Low	Low/normal	Low/normal	Absent
VWF:CB	Low (< 50%)	Low	Low	Low/normal	Low/normal	Absent
Ag/CB	Normal (0.5–1.5)	High (> 1.5)	High (> 1.5)	Normal/high	Normal (0.5–1.5)	Don't use
Ag/RCO	Normal (0.5–1.5)	High (> 1.5)	High (> 1.5)	Normal/high	Normal (0.5–1.5)	Don't use
CB/Ag	Normal (> 0.7)	Low (0–0.7)	Low (0–0.7)	Low/normal	Normal (> 0.7)	Don't use
RCO/Ag	Normal (> 0.7)	Low (0–0.7)	Low (0–0.7)	Low/normal	Normal (> 0.7)	Don't use

\*Absolute values noted in this table should be treated as a guide only; different laboratories will use different values based on internal or differentially published studies.

<sup>†</sup>Results depend on the specific mutation and defect defined; most cases identified to date show low VWF:Ag, lower (discordant) VWF:RCO, and low (but concordant) VWF:CB, with resultant low RCo/Ag but normal CB/Ag.

defect presenting in type 2B VWD is a heightened affinity of plasma VWF to platelet glycoprotein Ib/IX, leading to consequent loss of (primarily) HMW VWF from the circulation, and thus low RCo/Ag and low CB/Ag ratios are also typically evident. The level of specific "loss" of IMW and HMW VWF in type 2A VWD is generally greater than that for type 2B VWD, so that presenting ratios of RCo/Ag and CB/Ag are usually lower in type 2A VWD than type 2B VWD. However, this is an inconsistent finding, and these ratios cannot be used to confidently discriminate these two subtypes. In particular, it needs to be recognized that there is some considerable variability in the genetic and phenotypic profiles for both types 2A and 2B VWD. For example, the level of HMW VWF loss is somewhat variable between cases of type 2B VWD, as well as between sequential presentations within single individual cases. Although compared with type 2A VWD, type 2B also tend to show higher VWF:Ag levels and can also be associated with mild thrombocytopenia, there is some inconsistency in these findings, and so these cannot be used to confidently distinguish types 2A and 2B VWD. Nevertheless, such features can certainly help to guide the investigation. Similarly, VWF:multimers cannot distinguish type 2A from type 2B, although again, features may help guide the investigation. In practice then, testing of plasma from types 2A and 2B VWD will provide similar, or overlapping, phenotypic patterns (Table 1), and these VWD types can only be reliably distinguished using a RIPA procedure.

Type 2M VWD is a qualitative defect that presents with dysfunctional VWF but that is not associated with a loss of HMW VWF. Thus, the defect presenting in type 2M VWD is an inherent functional defect, and which defect presents depends on the genetic mutation evident. In practice, type 2M VWD would comprise a large composite of potential VWF defects that would not otherwise classify into the other VWD subtypes.<sup>8,9,11,12</sup> However, most 2M VWD defects so far described adversely affect the binding of plasma VWF to platelet glycoprotein Ib/IX (i.e., in contrast with the enhanced binding evident in type 2B VWD). Thus, most type 2M VWD defects so far described can be summarized as representing a platelet-binding dysfunction. Because the VWF:RCo assay is sensitive to these defects, these type 2M VWD defects typically present with relative low VWF:RCo values compared with VWF:Ag (i.e., low RCo/Ag). However, because these type 2M VWD mutations do not seem to so significantly influence the binding of VWF to matrix components such as collagen, VWF:CB test data instead tends to parallel VWF:Ag. Accordingly, most cases of currently type 2M classified VWD will present with low RCo/Ag but relatively normal CB/Ag, and this finding has led to many authors using this test pattern to "invariably" identify type 2M VWD, which is an inappropriate

oversimplification.<sup>11,12</sup> While this approach will in general identify such cases of (platelet-binding dysfunctional) type 2M VWD, it ignores all the other potential alternate type 2M VWD defects—including, but not limited to, genetic mutations that lead to loss of relative binding to matrix components such as collagen, but which otherwise have little affect on VWF binding to glycoprotein Ib/IX. These cases would still fit within a type 2M classification but would present with relatively low CB/Ag and feasibly normal RCo/Ag ratios. These type 2M VWD cases would also discriminate from types 2A and 2B VWD by not showing any specific loss of HMW VWF nor showing the enhanced RIPA characteristic of type 2B. Importantly, such cases have been reported<sup>25</sup> but may be relatively rare or perhaps less clinically severe when compared with the "more typically" reported (platelet-binding dysfunctional) type 2M VWD. Alternatively, the nonbalanced identification in these distinct type 2M VWD cases may merely reflect the fact that VWF:RCo assay has simply been an established assay for many more years and in many more laboratories than the VWF:CB assay, and possibly time and further study may redress the relative imbalance of differentially detected cases. Indeed, as a composite, the identification of many other putative type 2M VWD cases without loss of either VWF-collagen binding or VWF-platelet-glycoprotein-Ib/IX binding can be further hypothesized. We simply do not have all the possible tests available to identify all the possible VWF binding defects, but we do know that VWF binds to a multitude of other protein and nonprotein systems, so that in theory such possibilities are certainly plausible.

## OTHER SOLUTIONS AND STRATEGIES TO REDUCE AND LIMIT DIAGNOSTIC ERRORS

Identification of the low-level VWF sensitivity of laboratory assays is very important but possibly underappreciated. A laboratory cannot distinguish type 3 VWD from severe type 1 VWD, nor severe type 1 from type 2 VWD, unless the laboratory has knowledge of its assays' sensitivity limit. As previously mentioned, this is a particular problem with VWF:RCo assays.<sup>23,24</sup> There are several strategies to improve discrimination of VWF at low assay value ends. The first is to include a VWF-deficient plasma control in all assays. These plasmas are devoid of VWF and are commercially available. They should give a value of 0 in all VWF assays; if they give a value above 0, then the assay in question has a low-level VWF-sensitivity problem, and the level of VWF as defined by testing of this VWF-deficient plasma provides the lower level of sensitivity limit for the assay. The lower limit of sensitivity limit for VWF:Ag and VWF:CB assays is generally < 5% (it is 0 to 1% in our own laboratory); in contrast, the lower limit of sensitivity limit for VWF:RCo assays can be as



high as 15 to 25% (it is typically around 10% in our laboratory).<sup>23,24</sup>

Another strategy that laboratories can use to improve detection at low assay levels is to increase the concentration, or to lower the dilution, of test plasma used by the assay (i.e., individually tailor the test plasma dilution used to the plasma VWF level present). This is usually fairly easily achievable for VWF:Ag and VWF:CB assays, which typically use plasma dilutions

around 1:100, but is less often achievable for VWF:RCO assays, which use much lower plasma dilutions. The aim here is to test the plasma at a dilution that brings the derived test value within the ideal assay sensitive region. For VWF:Ag and VWF:CB assays, this is usually within the 20 to 100% VWF detection region. For example, a plasma sample containing a nominal (i.e., initially identified) level of 5% VWF should be retested at around 5 to 10 times the initial assay concentration used to bring it to

**Table 2 Laboratory Phenotypic VWF Assay Findings versus the Possibility or Otherwise of VWD\***

Assay Parameter	Laboratory Finding	Possibilities	Follow-up <sup>†</sup>
<b>VWF:Ag</b>	Normal (> 50%)	Not VWD, or type 2 VWD, or false high due to preanalytical event?	If clinical history is significant, assess VWF using functional assays to identify/exclude type 2 VWD, and repeat tests on new sample for confirmation.
	Low (< 50%)	VWD, or false low due to preanalytical event? If VWD, subtype cannot be identified without further testing.	If clinical history is significant, assess VWF using functional assays to identify/exclude type 2 VWD, and repeat tests on new sample for confirmation.
	Very low (< 10%)	Severe type 1 VWD, type 3 VWD, or type 2 VWD, or false result?	Assess VWF using functional assays to identify/exclude type 2 VWD, repeat tests on new sample for confirmation, identify low-level assay sensitivity properties to distinguish type 3 and severe type 1 VWD.
<b>VWF:RCO and VWF:CB</b>	Normal (> 50%)	Not VWD, or false high assay results (due to assay variation or preanalytical event)?	If clinical history is significant, repeat tests on new sample for confirmation.
<b>VWF:RCO and/or VWF:CB</b>	Low (< 50%)	VWD, or false low (due to assay variation or preanalytical event)?	If clinical history is significant, repeat tests on new sample for confirmation; review pattern of test results to help determine if type 2 VWD/which subtype.
<b>Ag/CB and Ag/RCO</b>	Normal (0.5–1.5)	Not VWD, or ?type 1 VWD, or possibly due to preanalytical event or assay variance?	If clinical history is significant, repeat tests on new sample for confirmation.
<b>Ag/CB and/or Ag/RCO</b>	High (> 1.5)	Type 2 VWD, or due to preanalytical event, or due to assay variance?	If clinical history is significant, repeat tests on new sample for confirmation; review pattern of test results and perform additional tests to properly subclassify type 2 VWD cases.
<b>CB/Ag and RCo/Ag</b>	Normal (> 0.7)	Not VWD, or ?type 1 VWD, or possibly due to preanalytical event or assay variance?	If clinical history is significant, repeat tests on new sample for confirmation.
<b>CB/Ag and/or RCo/Ag</b>	Low (0–0.7)	Type 2 VWD, or due to preanalytical event, or due to assay variance?	If clinical history is significant, repeat tests on new sample for confirmation; review pattern of test results and perform additional tests to properly subclassify type 2 VWD cases.

\*Absolute values noted in this table should be treated as a guide only; different laboratories will use different values based on internal or differentially published studies.

<sup>†</sup>As a general rule, the first thing to do as follow-up is to check the clinical history and the second is to repeat all tests using a fresh plasma sample tested a few weeks apart.



within this assay sensitive region, and this should therefore provide an interim value of around "25 to 50%," which can then be corrected by the adjusted dilution factor before being officially reported as 5%. If, for this example, however, the interim value obtained is significantly lower than the expected "25 to 50%," a lower-level-of-sensitivity issue might be present (e.g., an interim value of 5% obtained with a 10 × concentrated sample would actually correct to 0.5% and suggest that the originally identified level of 5% simply defined the lower limit of sensitivity for this assay).

Another way to help distinguish severe type 1, severe type 2, and type 3 VWD, if deemed appropriate, is to undertake a desmopressin (DDAVP) therapy trial (type 3 VWD will generally show no response, or no increase in plasma VWF after DDAVP), whereas severe type 1 VWD will show a small but detectable and similar rise in all VWF test parameters, and type 2 VWD should show a rise in VWF:Ag and correspondingly either a lower rise in functional VWF parameters (i.e., VWF:RCO and/or VWF:CB) or else a similar rise but more rapid disappearance because of faster clearance.

Additional VWF assay controls should include a type 2 VWD-like plasma to help identify the level of VWF assay discordance achievable within any given assay, and on both an intraassay and interassay basis. In our laboratory, this is simply achieved using cryosupernatant, obtained as a date-discarded blood-bank product and frozen in small aliquots for laboratory assay use. The characteristics of this plasma in our laboratory are now so well-known that we can identify assay problems very easily and very quickly. For example, the level of VWF:Ag detected is consistently between 10% and 20%, the VWF:CB level detected is consistently < 3%, and the CB/Ag ratio is consistently < 0.3. Results at variance to this can quickly identify assay problems that require rectification.

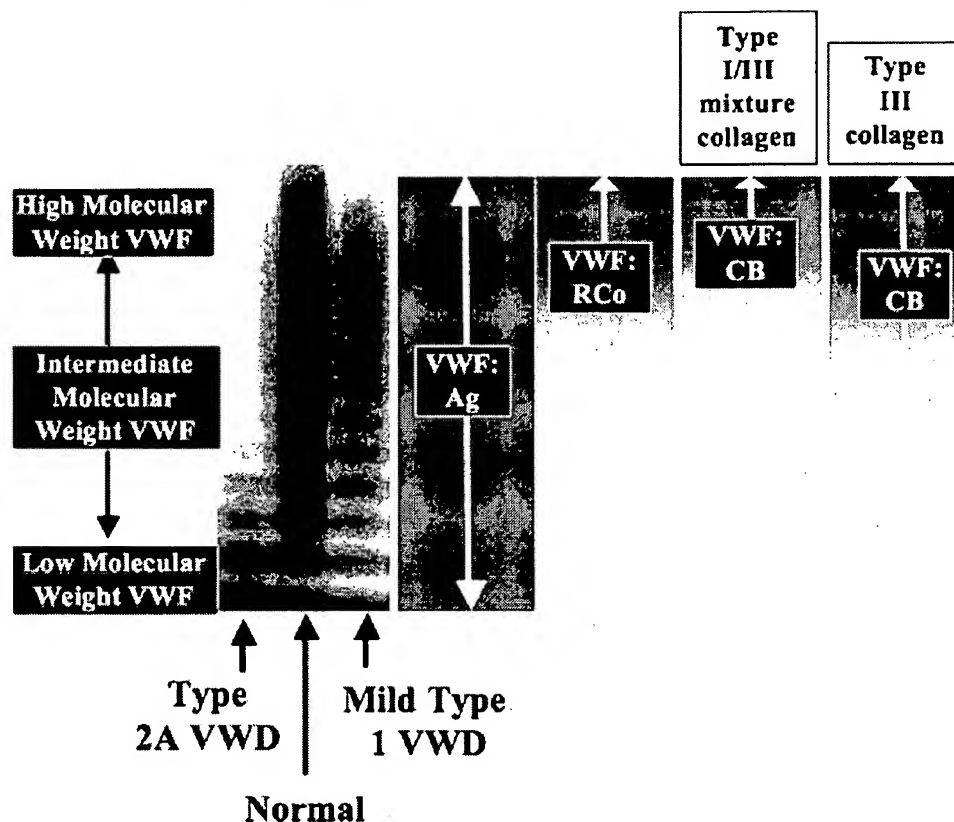
In addition to assay limitations, false abnormal VWF patterns can also arise from a variety of preanalytical events, most notably by provision of filtered plasma or postrefrigeration of whole blood.<sup>26,27</sup> As a blanket rule, patient follow-up should comprise a minimum of two events: (i) clinical history should be reevaluated to ensure that the test results match, and (ii) all tests should be repeated on a fresh sample taken some weeks apart for confirmation. Some other problems and potential follow-up processes are given in Table 2, which provides a summary of possible scenarios given certain VWD subtypes and testing patterns. For example, a low RCo/Ag *plus* low CB/Ag will usually reflect a type 2A or 2B VWD but might otherwise reflect a sample integrity issue. Similarly, a low RCo/Ag with normal CB/Ag may reflect a type 2M VWD or may otherwise reflect a false functional VWF discordance due to (VWF:RCO) assay variability. Laboratory testing realities are often under-recognized in clinical practice, and assay variabilities are

such that false-high and false-low high RCo/Ag ratios are not at all uncommon. This will respectively lead to false identification of type 1 VWD when testing type 2 VWD samples and false identification of type 2 VWD when testing type 1 VWD samples or normal samples. Although use of the VWF:CB is also capable of leading to such false test events, here the problem is generally less evident than that of the VWF:RCO, and the problem can be minimized by using a HMW sensitive (generally type I/III collagen mixture) VWF:CB assay.

### **WHAT TYPE OF COLLAGEN SHOULD WE USE FOR VWF:CB ASSAYS: TYPE I, TYPE III, OR A TYPE I/III MIXTURE?**

As mentioned, individual studies have shown purified type III collagen-based VWF:CB assays to be capable of some discrimination of HMW VWF and comparable with discrimination obtained using VWF:RCO assays.<sup>2-6</sup> Nevertheless, it has additionally been shown in *comparative* studies that type I/III collagen mixture-based VWF:CB assays tend to show even better discrimination of HMW VWF, and hence better discrimination of types 2A and 2B VWD (in particular) from types 1 and 3 VWD.<sup>2-5</sup> Moreover, it is clear from our own laboratory's very extensive studies that a type I/III collagen mixture, using a proportion of around 95%/5% type I/III collagen, respectively, works best in this capacity.<sup>2</sup> In our experience, a HMW-sensitive VWF:CB assay is significantly more sensitive to loss of HMW VWF than is a VWF:RCO assay. The perceived relationship between the molecular weight form of VWF and its identification using VWF:Ag, VWF:RCO, and different manifestations of VWF:CB assays is depicted in Fig. 1.

Nevertheless, most commercial manufacturers continue to supply purified type III collagen-based assays, and thus, most clinical studies that need to use a commercially sourced assay are "forced" to use potentially HMW-undersensitive VWF assays. Why do commercial manufacturers continue to use purified type III collagen-based preparations, despite evidence that type I/III collagen mixtures perform better in terms of HMW discrimination? One cannot be completely sure, as business interests would probably prevent them from telling us. However, I suspect that this is simply because type III collagen actually binds VWF much better than type I collagen, particularly in a static (nonshear) system such as the VWF:CB assay. Although this was something that I had come to well accept over the past 15 years, this has again most recently been highlighted by several reports on the interaction between VWF and collagen.<sup>28,29</sup> Accordingly, because of high-affinity binding, the use of purified type III collagen would provide VWF:CB assays with a greater amount of overall VWF adhesion and far better optical end-color generation in an ELISA system compared with one using a

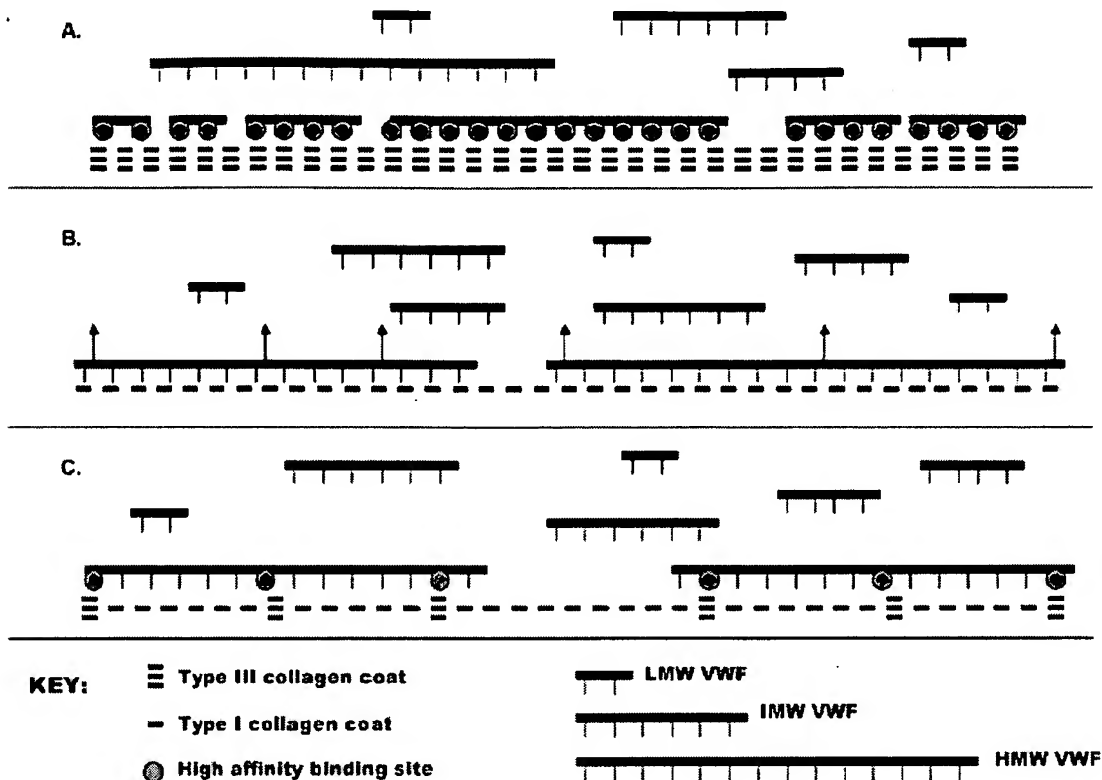


**Figure 1** Favaloro's VWF:CB hypothesis no. 1: "The use of different collagens in a VWF:CB assay affects its ability to discriminate high molecular weight (HMW) forms of VWF." The VWF:Ag assay detects all multimetric forms of VWF equally well (i.e., has no differential sensitivities to different molecular weight species including large, intermediate, and small multimers). The VWF:RCO assay detects all large and some of the intermediate VWF multimers. A fully optimized VWF:CB assay (using a type I/III collagen mixture of around 95%/5%, respectively) preferentially detects the hemostatically potent large VWF multimers. In contrast, a VWF:CB assay using purified type III collagen has a higher overall affinity for VWF and thus lower overall discrimination of HMW VWF; in the worst-case scenario, such an assay may end up detecting all multimetric forms of VWF (i.e., with no differential sensitivities to different molecular weight species), with performance more akin to a VWF:Ag assay. (Modified from Favaloro EJ. von Willebrand factor (VWF) collagen binding (activity) assay (VWF:CBA) in the diagnosis of von Willebrand's disorder (VWD): a 15-year journey. *Semin Thromb Hemost* 2002;28:191-202; Favaloro EJ. Laboratory identification of von Willebrand disease: technical and scientific perspectives. *Semin Thromb Hemost* 2006;32:456-471; Favaloro EJ. Laboratory monitoring of therapy in von Willebrand disease: efficacy of the PFA-100® and VWF:CB as coupled strategies. *Semin Thromb Hemost* 2006;32:566-576.)

type I/III collagen mixture. Purified type III collagen-based VWF:CB assays would also provide much better assay consistency (or precision), both within and between assays (i.e., would yield lower assay CVs, both intraassay and interassay). From a manufacturer assay-standardization and control-consistency viewpoint, purified type III collagen-based assays will currently win out over those based on type I/III collagen mixtures, simply because they are more "robust." However, commercial suppliers that manufacture VWF:CB kits using purified type III collagen, and the subsequently laboratories that use these products in clinical studies, seem to be missing a major point here; that is, the concept that a good ("optimized") VWF:CB assay needs to optimally

balance the *dual requirements* of an acceptable level of detectable binding of plasma VWF to collagen with that of optimal discrimination of HMW VWF.

So, yes, purified type III collagen binds VWF much better than type I collagen or even type I/III collagen mixtures. However, assays based on purified type III collagen are trading off the important property of "best" VWF binding against the critical property of "better" HMW discrimination. Thus, type III collagen binds VWF so well that assays based on purified type III collagen will simply also bind to the low-molecular-weight (LMW) forms of VWF. This is depicted in Fig. 2. At the other extreme end, it is likely that an assay based on purified type I collagen would bind VWF

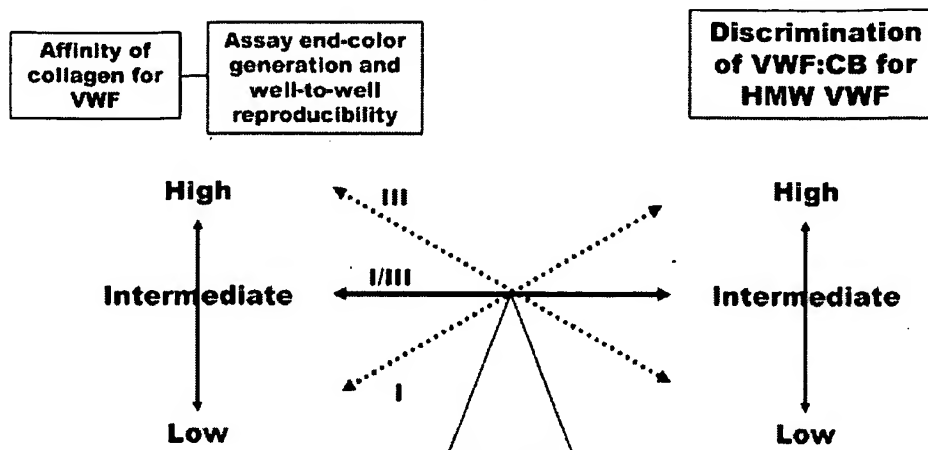


**Figure 2** Favaloro's VWF:CB hypothesis no. 2: "The ability of an optimized VWF:CB assay to both identify HMW VWF and produce acceptable assay performance in terms of end-color generation and well-to-well reproducibility results from a net balance of optimizing the *dual* requirements of detecting an acceptable level of binding of plasma VWF to collagen as well as the discrimination of HMW VWF by limiting the net affinity of this binding." The illustration shows simplified hypothetical representations of the binding of different molecular weight forms of VWF to collagen coated onto wells of an ELISA plate in a VWF:CB assay. Note that VWF will bind to collagen type III with high affinity but to collagen type I with low affinity. (A) All molecular weight forms of VWF might theoretically bind to ELISA wells coated with purified type III collagen, and the high-affinity binding will survive the rigors of an ELISA assay to generate a high degree of optical color generation, with excellent well-to-well reproducibility but comparatively poor HMW VWF sensitivity. (B) The poor VWF binding affinity of type I collagen will permit only the very HMW VWF forms to bind, with very few of these bound molecules able to survive the rigors of an ELISA assay—this would generate an assay with variable HMW sensitivity, a low and variable degree of optical color generation, and very poor well-to-well reproducibility. (C) In a type I/III collagen mixture-coated VWF:CB system, the high-affinity binding of VWF to type III collagen is moderated by the presence of type I collagen, which in essence acts like spacer arms; this would generate an assay with good HMW sensitivity but an intermediate degree of optical color generation and intermediate well-to-well reproducibility. Although acceptable performance can be obtained after assay optimization, it is doubtful that the latter two properties can match those of a purified type III collagen-based VWF:CB, and this will raise issues for commercial kit manufacturers. On the other hand, it is unlikely that the HMW sensitivity of a type I/III collagen mixture-based VWF:CB will be matched by an assay based on purified type III collagen.

very poorly; so poorly that only the very HMW forms of VWF would bind. This would theoretically yield an assay highly sensitive to HMW VWF. However, because type I collagen binds VWF so poorly, very little of the VWF initially attached will likely survive the rigors of the ELISA assay. Hence, this would not provide for a very robust assay and would ultimately yield poor end-color generation and very poor reproducibility. VWF:CB assays based on type I/III collagen mixtures will provide something in between, and an optimized VWF:CB assay is one that is optimized for discrimination of HMW VWF, but at the same time has

acceptable (but perhaps not perfect) characteristics of VWF binding that ultimately permits acceptable (but perhaps not perfect) optical visualization (i.e., end-color generation) and acceptable (but perhaps not perfect) assay reproducibility (Fig. 3).

It is interesting that a mixture of around 95% type I/5% type III collagen seems to work best for this dual requirement.<sup>2</sup> Moreover, it is actually quite likely that most of the VWF binding in the type I/III collagen mixture-based assay is actually accomplished via the type III collagen. The type I collagen may simply act like "spacer arms," to reduce the overall interaction of the



**Figure 3** Favaloro's VWF:CB hypothesis no. 3: "The affinity of a collagen to VWF is inversely proportional to its ability to discriminate HMW VWF." The illustration shows a simplified hypothetical representation of the relationship between the affinity of collagen binding to VWF, the ability to obtain end-color generation and well-to-well reproducibility, and the ability to discriminate HMW VWF. High-affinity binding of type III collagen will result in high end-color generation and high well-to-well reproducibility but relatively poorer HMW VWF discrimination. Low-affinity binding of type I collagen will result in HMW VWF discrimination but relatively poor or highly variable high end-color generation and well-to-well reproducibility. An appropriately optimized VWF:CB assay using a type I/III collagen mixture will balance these properties to generate an assay that has acceptable HMW VWF discrimination and acceptable end-color generation and well-to-well reproducibility.

presenting VWF to predominately that with type III collagen (as depicted in Fig. 2). What this means in practice, then, is that LMW forms of VWF cannot bind to the collagen, or if they do bind, they bind with low affinity to the predominant type I collagen, and after assay equilibrium would either be replaced by the HMW VWF forms or else would be washed away during the ELISA procedure. In contrast, HMW VWF *can* bind, and the larger the VWF molecule, the more likely it is able to bridge several type III collagen molecules and to also withstand the rigors of the ELISA assay (hence the ability of a type I/III collagen mixture-based VWF:CB to discriminate HMW VWF). It is also likely that any success so far attributed to the use of "purified" type I collagen-based VWF:CB assays is actually due to small amounts of "contaminating" type III collagen—after all, even 1% "contaminating" type III collagen will provide some significant additional VWF binding to such an assay. Although type III collagen will also likely bind HMW VWF better than LMW VWF (and hence assays based on purified type III collagen will have *some* HMW discriminatory capacity), the comparative proportion of HMW/LMW VWF bound within a given VWF:CB assay is likely to be higher in a more selective VWF:CB assay (i.e., using a type I/III collagen mixture product).

So, let us paraphrase the above one last time for emphasis: what laboratories require to discriminate HMW VWF forms *is* an assay that binds VWF, but one that binds VWF only *moderately* well, so that it will *preferentially* bind only the largest and most adhesive forms of VWF. Hence our ongoing recommendation for

the selection of a type I/III collagen mixture, as this seems to work best. Indeed, laboratories already have assays that bind VWF extraordinarily well—they are called VWF:Ag assays.

### THUS, CAN THE VWF:CB ASSAY REPLACE THE VWF:RCO ASSAY, AND IF NOT, WHY NOT?

I have mentioned that this is one of the questions that I am most regularly asked, and the simple answer is no, the VWF:CB assay cannot replace the VWF:RCo assay per se. However, if used as a part of a laboratory test panel and with the right strategies in place, the VWF:CB assay can replace some of the need to perform VWF:RCo testing. As mentioned at the beginning of this article, our laboratory originally investigated the utility of the VWF:CB assay with the view to potentially replacing our laborious and highly variable VWF:RCo assay. Some 15 years on, we continue to use the VWF:RCo assay, albeit not for all patient investigations. There are two main reasons we still retain the VWF:RCo assay: first, despite its previously noted limitations (time intensive, poor assay reproducibility, poor low VWF level sensitivity), it remains a useful assay to confirm VWF dysfunction in type 2 VWD such as types 2A and 2B VWD, and second, it is a critical assay should the laboratory wish to identify or discriminate the main (platelet-binding dysfunction) presentation of type 2M VWD. We need to remember that the VWF:RCo and VWF:CB do not detect the

same adhesive VWF function; the former reflects the functional or adhesive ability of VWF to bind to platelets (via the glycoprotein Ib/IX receptor), and the latter reflects the functional or adhesive ability of VWF to bind to subendothelial matrix components (i.e., collagen used as surrogate). Thus, although each assay can be used to help identify the loss of HMW VWF that might be present in types 2A and 2B VWD or to identify the presence of HMW VWF that might be normally present in normal plasma or plasma from type 1 VWD, each assay also has the potential to differentially identify different inherent VWF defects presenting in type 2M VWD.

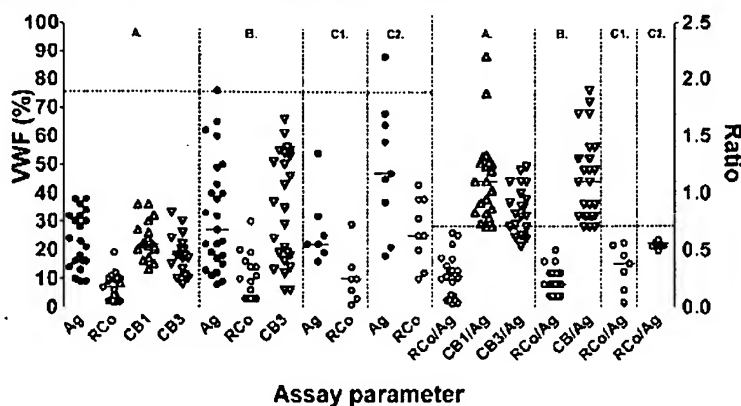
Although we do not consider it appropriate to replace the VWF:RCO assay with the VWF:CB testing *per se*, at the same time we no longer consider it appropriate to use only the VWF:RCO assay as the sole functional phenotypic VWF assay. It has already been mentioned that the VWF:RCO assay has high relative intraassay and interassay variability and that this variability is generally greater than that observed for VWF:CB assay. It has also been noted that the consequence of using the combination of VWF:Ag and VWF:RCO as the only VWF assays to screen for the possibility of VWD is that a significant number of "true-positive" individuals will be missed or misidentified and several "false-positives" will also be identified, and that adding the VWF:CB assay to the test panel will reduce these diagnostic errors. However, we now additionally need to recognize the probability of VWD mutations that will differentially be identified by testing with VWF:RCO assay *or* with VWF:CB assay.<sup>8,11,12</sup> Most of these will sit within a type 2M VWD classification. Most cases of type 2M VWD so far described are those that show an inherent defect of VWF binding to platelet glycoprotein Ib/IX and hence have been identified from discordance in VWF:RCO to VWF:Ag (i.e., low RCo/Ag ratio, with fairly normal CB/Ag if VWF:CB testing also performed). Although cases of type 2M VWD characterized with abnormal VWF binding to collagen (i.e., low CB/Ag) but with fairly normal VWF binding to platelet glycoprotein Ib/IX (i.e., normal RCo/Ag) have been described, this is a rare finding. What is not known is if these are truly rare cases or whether these cases fail to be identified simply because the more typically used screening panel of VWF:Ag and VWF:RCO assays would merely fail to identify them.

Although we would prefer to use the more comprehensive test panel of VWF:Ag, VWF:CB, FVIII:C, and VWF:RCO assays to screen *all* cases being investigated for VWD, testing and staffing realities prevent this from occurring in practice within most laboratories. Indeed, of 55 current participants of the external quality assurance program (QAP) that is most used within our geographic locality (the RCPA QAP), most do not perform very comprehensive test panels.<sup>10,16</sup> This is

despite most of these laboratories being actively involved in testing patient samples for possible VWD, and despite the RCPA QAP having the largest proportion of VWF:CB testing laboratories (around 50% of participants) when compared with other comparable QAPs (which range from < 5% in the United States to some 25% in Europe).<sup>16</sup> Around 20% of current RCPA QAP participant laboratories have a very limited test panel (e.g., only FVIII:C and VWF:RCO [*n* = 1], or FVIII:C and VWF:Ag [*n* = 10]). Furthermore, although around 80% of RCPA QAP participant laboratories perform VWF:Ag, FVIII:C, and at least one VWF functional assay, the actual VWF panel used varies widely. The VWF test panel of VWF:Ag and VWF:RCO only is used by 11 (21.2%) laboratories, VWF:Ag and VWF:CB only by 5 (9.6%) laboratories, and VWF:Ag, VWF:RCO, and VWF:CB used by 16 (30.7%) laboratories. In other words, despite the VWF test panel of VWF:Ag, VWF:RCO, and VWF:CB being that currently preferred, less than a third of laboratories in our geographic locality do so, with most laboratories either replacing the VWF:RCO with another functional VWF assay such as VWF:CB or else abandoning the assay altogether. Although comparative data from other geographic localities is lacking, we might expect that such trends will most likely continue elsewhere, given the noted issues with VWF:RCO testing, and the tempting comparative ease of testing using potentially "alternate" functional VWF assays.

It has already been noted that a VWF test panel including the VWF:CB will tend to misidentify cases of type 1 or type 2 VWD far less often than a VWF test panel using VWF:RCO only as functional VWF assay. However, a VWF test panel of VWF:Ag and VWF:CB might potentially miss platelet-binding dysfunctional type 2M VWD cases. At least it will, in theory; in practice, our current view is that a VWF test panel of VWF:Ag and VWF:CB *can* be used to *screen* for such type 2M VWD cases, as all cases of type 2M VWD so far reported have shown VWF:Ag *and/or* VWF:CB levels of < 75%. Accordingly, if a laboratory determines a consistent finding of VWF:Ag *and* VWF:CB of say > 75%, then this finding could be used with a high degree of confidence to exclude type 2M VWD.

This was our laboratory's view after a review of the literature as of 2002<sup>4,11</sup> and still remains our view after an update of the literature review. In brief, there are several individual reports of type 2M mutations, as well as several large-scale studies limited to phenotypic testing with VWF:Ag and VWF:RCO. More recently, however, several large-scale studies have also been reported that have included genetic testing, plus phenotypic testing with VWF:Ag, VWF:RCO, and in most cases VWF:CB. The available data has been replicated in Fig. 4. As can be seen, when reported, VWF:CB values have invariably been < 75% and are much more typically reported in the



**Figure 4** Summary of recently published phenotypic data from genetically confirmed cases of type 2M VWD showing reported results, where available, for VWF:Ag, VWF:RCo, and VWF:CB assays plus ratios of CB/Ag and RCo/Ag. CB1 indicates a VWF:CB based on type I collagen (or ?type I/III collagen mixture); CB3 indicates a VWF:CB based on type III collagen. (Region C1 shows data for cases with mutation found and confirming type 2M VWD; region C2 for remaining cases with no mutation found but where type 2M VWD was thought feasible given phenotypic data). No case of confirmed type 2M VWD has ever been identified with VWF:Ag and VWF:CB below 75%. (Data from Penas N, Pérez-Rodríguez A, Torea JH, et al. Willebrand disease R1374C: type 2A or 2M? A challenge to the revised classification. High frequency in the northwest of Spain [Galicia]. *Am J Hematol* 2005;80:188–196 [region A of figure]; Riddell AF, Jenkins PV, Nitu-Whalley IC, McCraw AH, Lee CA, Brown SA. Use of the collagen-binding assay for von Willebrand factor in the analysis of type 2M von Willebrand disease: a comparison with the ristocetin cofactor assay. *Br J Haematol* 2002;116:187–192 [region B of figure]; James PD, Notley C, Hegadorn C, Poon MC, Walker I, Rapson D. Association of Hemophilia Clinic Directors of Canada, Lillicrap D. Challenges in defining type 2M von Willebrand disease: results from a Canadian cohort study. *J Thromb Haemost* 2007;5:1914–1922 [regions C1 and C2 of figure].)

abnormal assay range. VWF:Ag data are also typically reported in the abnormal assay range, although occasionally normal. Although there have been two isolated reports of VWF:Ag above 75%, there has never been a single case of platelet-binding dysfunctional type 2M VWD described with values of VWF:Ag and VWF:CB above 75%. Moreover, in the comprehensive type 1 VWD study reported by Goodeve et al, the group of cases that could potentially be redefined as type 2 VWD yielded VWF:Ag values in the range 5 to 74%, and VWF:CB values in the range 4 to 23%.<sup>18</sup>

### CAN THE VWF:CB ASSAY BE USED TO ABROGATE THE NEED TO PERFORM MULTIMER TESTING?

Interestingly, although the VWF:CB assay has not replaced the VWF:RCo assay within our laboratory, it has instead largely abrogated the need to perform routine multimer analysis in the vast majority of cases under investigation. That is, the VWF:CB assay, specifically sensitive to the presence of HMW VWF, as part of a comprehensive test panel inclusive of FVIII:C, VWF:Ag, and VWF:RCo, and RIPA testing and/or VWF:FVIII:B where indicated, can usually replace the need to perform VWF:multimer testing, as such testing will generally just act to confirm the findings of the previously performed phenotypic tests and will not otherwise better or more definitively characterize most patients, nor will the outcome of

multimer investigation usually influence differential clinical management.

This is generally true of:

- Type 1 VWD, which can be appropriately diagnosed by reproducible low but concordant levels of plasma VWF using the three phenotypic assays (i.e., VWF:Ag plus VWF:CB plus VWF:RCo all low and concordant on several test occasions); performance of multimers will simply be a confirmative assay and show reduced intensity with normal banding patterns.
- Type 2 VWD, which is generally identified by discordance in plasma phenotypic patterns (i.e., VWF:Ag plus VWF:CB and/or VWF:RCo for types 2A, 2B, and 2M VWD; VWF:Ag plus FVIII:C for type 2N VWD). Although types 2A, 2B, and 2M might give perceptually similar patterns in phenotypic studies (i.e., discordance of VWF:CB and/or VWF:RCo with VWF:Ag), type 2B can be readily distinguished from types 2A and 2M by performing a RIPA assay and identifying low-dose ristocetin responsiveness. Type 2A VWD cannot be conclusively distinguished from type 2B VWD by using a multimer assay. Although types 2A and 2M VWD can be distinguished by multimer analysis, such discrimination is generally clinically irrelevant in regard to therapy and management, and as stated previously, most cases of type 2M VWD are platelet-binding dysfunctional and hence tend to also show low RCo/Ag but reasonably normal CB/Ag, whereas type 2A

VWD will tend to show both low RCo/Ag and low CB/Ag. Types 2A and 2M VWD can also be distinguished from type 1 VWD, which will show normal RCo/Ag and normal CB/Ag. We would propose a DDAVP trial might be more beneficial than performance of multimers for the further investigation and characterization of possible types 2A, 2M, and 1 VWD, as it will help both better characterize the defect and also provide information regarding possible therapeutic approaches and their limitation. Finally, multimer assessment will not help identify type 2N VWD, which requires performance of a specific VWF:FVIII assay.

- Type 3 VWD, which can be appropriately diagnosed by reproducible absence of detected plasma VWF using the three phenotypic assays (i.e., VWF:Ag plus VWF:CB plus VWF:RCo all show an absence of VWF and this result is reproducible on several test occasions); performance of multimers will simply be a confirmative assay and show a similar absence.

This viewpoint is supported by a retrospective assessment of comparative multimer patterns against phenotypic patterns in our laboratory using a 2-year data period where we performed testing for FVIII:C, VWF:Ag, VWF:RCo, and VWF:CB and multimer analysis on all samples received for testing, and where we identified that multimer patterns could invariably have been predicted from the presenting phenotypic patterns.<sup>30</sup> A similar but numerically larger recent study from the United States has found similarly, and that performance of VWF:CB as part of a panel of tests was able to negate the need to perform multimer analysis in the vast majority of test cases.<sup>31</sup> Nevertheless, although this was the case for these laboratories, it is important to recognize that different geographic localities and patient referral patterns could give rise to different conclusions. If patients have a significant history of mucocutaneous bleeding, and if the investigations using the standard test repertoires are found to be "normal," inclusive of blood counts and morphology, VWF and clotting factor testing, plus platelet function testing, then multimer testing could be warranted. It is not doubted by this author that we will continue to discover variant forms of clinically significant VWD with multimer banding pattern abnormalities reflective of some diminished VWF function but otherwise normal using the currently standard VWF test repertoire. Just as we can define VWF abnormalities characterized as type 2M VWD and identified only by abnormal VWF:RCo or abnormal VWF:CB, it can be predicted that we will ultimately also identify VWF abnormalities detectable only from abnormal VWF multimer profiles. However, these would likely be rare, and for the vast majority of patient investigations, multimer analysis can be omitted (assuming the laboratory had access to the battery of phenotypic tests previously noted).

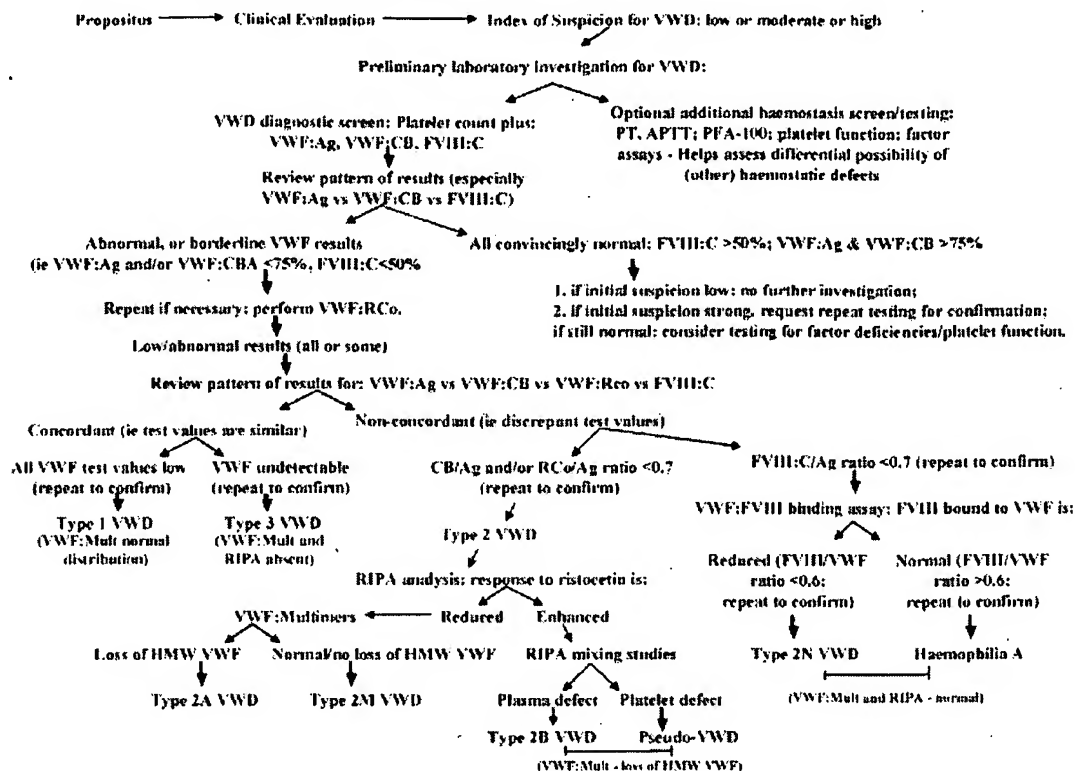
## THE LABORATORY EVALUATION OF VWD: ONE LABORATORY'S APPROACH

Although this has been highlighted in several reviews, a brief synopsis here might be of final additional value and follows on from the above discussion. Our initial approach to the laboratory investigation of VWD in a given patient is generally dependent on three main points: (1) the level of clinical and family history made available to us, (2) whether the patient is referred to us by a hematologist versus a non-hematology specialist or generalist clinician, and (3) whether the patient presents to us personally or else is sent to us as a referred plasma sample. We tend to take investigations further when they present to us personally or when they are referred to us by hematology specialists and/or have convincing clinical histories inclusive of strong personal histories of mucocutaneous bleeding. In this case, we have a greater incentive to investigate more extensively as well as having access to whole blood, and thus our standard plasma-based VWF test protocol might be supplemented with blood counts and morphology, blood grouping, and platelet function testing (either classic aggregation, PFA-100 closure time, or both, depending on the history, sample availability, and time availability; Fig. 5). For subsequent investigation, or for investigation of frozen plasma samples referred to us, our standard plasma-based VWF test protocol is employed, with follow-up suggestions depending on laboratory findings. Our standard VWF test protocol comprises FVIII:C, VWF:Ag, and VWF:CB testing. If no clinical history is provided to us, and all test results are convincingly normal (i.e., > 75%), then no further testing is performed by us (including VWF:RCo). The rationale here is that such convincingly normal (i.e., > 75%) test results should exclude all currently identifiable cases of VWD, including all types 1, 2A, 2B, 2M, 2N, and 3.

If any assay value falls below 75%, then there is a need to perform some follow-up. If the level of VWF detected by VWF:Ag and VWF:CB is significantly low but proportionally similar, then type 1 VWD or type 2M VWD cannot be excluded and VWF:RCo is performed (to help discriminate types 1 and 2M VWD), and repeat testing is also recommended. If VWF:Ag and VWF:CB assay results are discordant, a type 2A or 2B VWD defect is possible, and this also requires some follow-up, including VWF:RCo assay, repeat testing, and possibly RIPA analysis. If FVIII:C is proportionally lower than VWF, then either hemophilia A or type 2N VWD is possible and should be investigated/discounted (a family history is needed, repeat testing may be useful, and a VWF:FVIII assay may also be required).

Remember that if VWF:RCo is discordant to VWF:Ag, but VWF:CB concordant to VWF:Ag, then we have a case of either platelet-binding dysfunction type 2M VWD or of false RCo/Ag discordance (a family





**Figure 5** An algorithm that describes our laboratory's current procedure for VWD investigation. An appropriate clinical history is paramount. If time is critical, and the patient investigation new, then simultaneous performance of additional tests (e.g., routine coagulation tests such as prothrombin time (PT) and activated partial thromboplastin time (APTT), full blood count, and a platelet function screen such as the PFA-100) may also be warranted to assess the likelihood of other hemostatic disorders or thrombocytopenia. This algorithm provides a general guide only; all patients are individuals and should be treated as such. This VWD investigation process should enable the initial identification of all forms of VWD and at conclusion an appropriate classification.

history is needed and repeat testing may be useful). Although multimers can be performed to distinguish types 2A and 2M VWD, this can usually be omitted as these patients are generally treated identically by clinical therapy. A DDAVP trial might be useful to help distinguish these VWD subtypes but will often show limited therapeutic utility, and VWF concentrates are typically employed to manage these patients. As already mentioned, most cases of type 2M VWD are platelet-binding dysfunctional and hence will tend to show low RCo/Ag but reasonably normal CB/Ag, whereas type 2A VWD will tend to show both low RCo/Ag and CB/Ag, and type 1 VWD will tend to show normal RCo/Ag and CB/Ag.

#### UTILITY OF THE VWF:CB ASSAY FOR MONITORING THERAPY IN VWD

This has been the subject of several articles from our laboratory and will thus only be briefly summarized here for completeness. We propose that the VWF:CB assay,

in conjunction with the VWF:RCo and VWF:Ag assays, may provide additional useful pharmacokinetic data in future studies evaluating the clinical utility of VWF concentrates.<sup>32,33</sup> We also propose the potential utility of CB/Ag and RCo/Ag ratios as providing markers of delivered VWF functionality in such studies. We also propose that the VWF:CB assay and ratios of CB/Ag and RCo/Ag be given consideration for labeling of VWF concentrates used for therapy in VWD.<sup>32-34</sup> Finally, we propose that the VWF:CB is a useful marker of DDAVP responsiveness in patients given this drug as therapy.<sup>35-37</sup>

#### STANDARDIZATION ISSUES AND CONCLUDING COMMENTS

Standardization of VWF:CB assays has also been extensively discussed in previous articles from our laboratory.<sup>4,38</sup> Our laboratory continues to drive toward standardization of VWF:CB assays using type I/III



collagen mixture-based products for VWD diagnostics for the reasons previously noted, and this also appears to the view of other experts.<sup>5,39</sup> With respect to factor concentrate testing, labeling, and standardization, we would also support standardization using a type I/III collagen-based VWF:CB assay, and again this also appears to the view of other experts.<sup>5,40,41</sup> We reiterate that the use of different collagen sources as coating material for VWF:CB assays plays a critical part in this assay's ability to discriminate HMW forms of VWF or else to fail to discriminate these forms.<sup>2</sup> Standardization of VWF:CB assays should be toward an assay that both discriminates HMW forms of VWF as well as provides an adequate framework to permit a sufficient overall level of VWF binding that can be confidently and consistently determined on an assay-to-assay basis. We hope that the evidence and our arguments in support of a type I/III collagen mixture are convincing enough to find favor with clinical laboratories, the commercial manufacturers, and their regulators. For commercial manufacturers, we need to be persuasive to both the manufacturers of the VWF:CB assay kits and the manufacturers of VWF concentrates. Otherwise, both may continue to have a preference for, or otherwise show a bias toward, the use of purified type III collagen-based VWF:CB assays.<sup>33,38</sup>

#### ABBREVIATIONS

Ag/CB	ratio of VWF:Ag to VWF:CB
Ag/RCo	ratio of VWF:Ag to VWF:RCo
APTT	activated partial thromboplastin time
CB/Ag	ratio of VWF:CB to VWF:Ag
CV	coefficient of variation
DDAVP	desmopressin
ELISA	enzyme-linked immunosorbent assay
HMW	high molecular weight (VWF)
IMW	intermediate molecular weight (VWF)
LIA	latex immunoassay
LMW	low molecular weight (VWF)
PT	prothrombin time
RCo/Ag	ratio of VWF:RCo to VWF:Ag
RIPA	ristocetin-induced platelet agglutination
VWD	von Willebrand disorder/disease
VWF	von Willebrand factor
VWF:Ag	von Willebrand factor antigen
VWF:CB	von Willebrand factor collagen binding capacity
VWF:multimers	multimer assessment or structural profile for VWF
VWF:RCo	von Willebrand factor ristocetin cofactor activity

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# Laboratory Tests for Measurement of von Willebrand Factor Show Poor Agreement among Different Centers: Results from the United Kingdom National External Quality Assessment Scheme for Blood Coagulation

Steve Kitchen, Ph.D.,<sup>1</sup> Ion Jennings, Ph.D.,<sup>1</sup> Tim A.L. Woods,<sup>1</sup> Dianne P. Kitchen,<sup>1</sup> Isobel D. Walker, M.D.,<sup>1</sup> and Francis E. Preston, M.D.<sup>1</sup>

## ABSTRACT

In recognition of the importance of von Willebrand factor (vWF) testing in the diagnosis of von Willebrand disease (vWD), the United Kingdom National External Quality Assessment Scheme for Blood Coagulation regularly distributes samples for determination of vWF:antigen (vWF:Ag). Data from 10 separate surveys performed between 2001 and 2005 are reviewed. These include results from ~200 different centers, of which 55% are within the United Kingdom and the remainder are from other countries. During the period of the surveys, the use of immunoelectrophoresis for determination of vWF:Ag practically disappeared and was largely replaced by latex agglutination assays. The coefficient of variation (CV) of results in different centers was ~15 to 20% for most vWF:Ag techniques, with CVs of ~7% for a fluorescence-based assay. Several different techniques were used for determination of vWF ristocetin cofactor activity (vWF:RCo), all of which were associated with poor agreement among centers as indicated by CVs of 40 to 50%. Several centers calculated the ratio of vWF:Ag/vWF:RCo but with variable success. Ratios compatible with either type 1 or type 2 vWD were obtained on samples from subjects with type 1 vWD, as well as on samples from subjects with genetically confirmed type 2 vWD. Overall, our data show that laboratory testing for vWD remains problematic. It remains to be seen whether newer techniques will offer consistently improved precision.

**KEYWORDS:** von Willebrand factor:antigen (vWF:Ag), von Willebrand factor:ristocetin cofactor activity (vWF:RCo), laboratory assays, von Willebrand disease (vWD)

Von Willebrand disease (vWD) is considered to be the most common inherited bleeding disorder,<sup>1</sup> and

yet in many national registries the number of diagnosed patients is less than the number of patients diagnosed

<sup>1</sup>United Kingdom National External Quality Assessment Scheme (NEQAS) for Blood Coagulation, Sheffield, United Kingdom.

Address for correspondence and reprint requests: Steve Kitchen, Ph.D., United Kingdom National External Quality Assessment Scheme (NEQAS) for Blood Coagulation, Rutledge Mews, 3 Southbourne Road, Sheffield, [postal code] United Kingdom. E-mail: Steve.Kitchen@sth.nhs.uk

Laboratory Issues in the Identification and Diagnosis of von

Willebrand Disease. On the 80th Anniversary of Erik von Willebrand's Original Publication; Guest Editors, Emmanuel J. Favaloro, Ph.D., M.A.I.M.S., and Jan J. Michiels, M.D., Ph.D.

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DOI 10.1055/s-2006-947863. ISSN 0094-6176.

with hemophilia A. This anomaly, in part, relates to problems with the laboratory tests used in the diagnosis of vWD. Phenotypic assays for the presence of vWF measured as vWF:antigen (vWF:Ag) and assays of the function of vWF either as ristocetin cofactor activity (vWF:RCO) or more recently as collagen binding (vWF:CB) remain the tests of choice to screen for possible vWD.<sup>1-3</sup> Classification into type 1, 2, or 3 vWD, and further division of type 2 vWD into subtypes, has important implications for clinical management.<sup>4</sup> In this respect, molecular analysis of DNA is useful for many but not all type 2 vWD patients,<sup>1,5</sup> and is not yet widely used in diagnosis of type 1 vWD.

Furthermore, in patients with symptomatic bleeding, determination of vWF levels is reasonable, given that a low level of vWF is a risk factor for bleeding, although a low level does not mandate a diagnosis of vWD.<sup>6</sup> In recognition of the importance of measuring vWF levels, the United Kingdom National External Quality Assessment Scheme (UK NEQAS) regularly distributes samples to participating laboratories performing these investigations. A large database of participants allows assessment of the between-center agreement as well as evaluation of the relative merits of the different tests currently used in service departments dealing with the investigation of patients with symptomatic bleeding.

## MATERIALS AND METHODS

### UK NEQAS Surveys

Data from ten UK NEQAS surveys conducted between 2001 and 2005, distributed at approximately six-month intervals, are reviewed. In each survey a lyophilized plasma sample was distributed to participating centers that were invited to determine vWF:Ag and vWF activity by the methods in routine use for patient investigation. For analysis, results were grouped according to the method used. The median and coefficient of variation (CV) were calculated for each method group, without exclusion of any data points.

### Samples and Patients

A total of 10 samples were prepared; nine of these were from individual patients previously ascribed a diagnosis of type 1 or type 2 vWD. Samples were collected from the following: type 1 vWD, four different samples from two patients; type 2M vWD, three different samples from the same patient; and type 2N, two different samples from one patient. vWD had been diagnosed previously according to United Kingdom Haemophilia Centre Doctors' Organisation guidelines<sup>1</sup> based on multiple blood samples collected on several different occasions. Subjects with type 2 vWD based on vWF:Ag,

vWF:RCO, and vWF multimeric analysis were investigated using polymerase chain reaction, confirmation-sensitive gel electrophoresis, and DNA sequences as described previously.<sup>5</sup> The amino acid substitutions were as follows: type 2M, Arg1374Cys; and type 2N, Arg854Gln. The Arg1374Cys defect has been reported to be associated with type 2M/2A vWD.<sup>7</sup> The subject in our study had normal vWF multimers (consistent with type 2M vWD). In addition, the Secondary Standard for Coagulation/International Society on Thrombosis and Haemostasis, prepared from a pool of normal donors, was distributed as a normal plasma.

Approximately 600 mL of plasma was collected from each donor by plasmapheresis into citrate-phosphate dextrose anticoagulant during 40 to 45 minutes. Each donation was then centrifuged twice at 2500 × g for 30 minutes at 4°C, leaving a residual platelet count of less than 10 × 10<sup>9</sup>/L. This was then buffered by addition of 3.4 mM hydroxyethylpiperazine ethane sulphonic acid, frozen in 0.5-mL aliquots in siliconized glass vials and lyophilized for 6 days. Lyophilized samples were stored at -20°C prior to despatch through the mail to participants. Each sample was analyzed in a different survey.

## RESULTS

### vWF:Ag

From 2001 to 2005, the number of centers participating in the vWF:Ag surveys increased from 159 (71% from within United Kingdom) to 186 (54% within United Kingdom). There were marked changes in the number of centers using some techniques. Use of immunoelectrophoresis (Laurell rockets) decreased from 14% of centers in 2001 to 1% in 2005. During this time the use of latex-based immunoturbidometric assays increased from 14% of centers to 61% of centers. Enzyme-linked immunosorbent assay (ELISA) users decreased from 48% to 26%.

The results obtained by users of ELISA (at least nine different antibody sources), the immunoturbidometric assays from Instrumentation Laboratory (IL; Lexington, MA) and Liatest (Stago; Paris, France) are shown in Table 1. These were the most widely used methods in the period of study. There were no statistically significant differences between the median results obtained by users of these three techniques. The mean CV for ELISA users was 20.5% compared with 18% for Stago Liatest and 14.4% for the IL immunoturbidometric assay (not significant). The mean CV for immunoelectrophoresis in the five surveys for which at least 10 centers used this method was 26.6%. An enzyme-linked fluorescence assay (ELFA) was used by more than 10 centers in three survey distributions; the mean CV for results from these centers was 7.1%.

**Table 1 Results of vWF:Ag Assays Grouped According to the Method in Use: UK NEQAS Participants Data 2001–2005**

Date of Survey	Sample Type	Number of Users			Median vWF:Ag (IU/dL)			CV (%)		
		ELISA	IL*	Stago†	ELISA	IL	Stago	ELISA	IL	Stago
January 2001	Type 2M	70	13	20	16.0	15.5	17.0	27.0	10.7	17.8
September 2001	Type 1	67	20	27	29.0	30.6	28.9	26.6	17.7	13.1
November 2001	Normal	67	31	36	97.0	97.4	98.0	9.7	7.5	27.6
May 2002	Type 2N	59	32	35	96.5	107.0	103.0	14.2	9.1	12.3
November 2002	Type 1	57	37	37	28.5	28.0	29.7	14.8	5.3	12.1
May 2003	Type 1	52	37	38	29.0	27.7	30.9	17.3	13.3	9.7
November 2003	Type 2M	52	41	39	10.0	10.0	12.0	34.1	25.6	30.1
May 2004	Type 2N	47	43	43	108.0	105.0	115.2	18.9	9.2	9.8
November 2004	Type 2M	46	47	40	10.8	10.0	11.8	23.6	29.3	36.9
May 2005	Type 1	47	49	44	16.4	16.0	18.4	19.2	15.8	11.8
Mean	—	56	35	36	43.1	44.7	46.5	20.5	14.4	18.1

\*Instrumentation Laboratory immunoturbidometric assay.

†Stago Liatest immunoturbidometric assay.

vWF:Ag, von Willebrand factor:antigen; UK NEQAS, United Kingdom National External Quality Assessment Scheme; ELISA, enzyme-linked immunosorbent assay; CV, coefficient of variation.

#### vWF:RCo and vWF Activity

The number of centers returning results for vWF:RCo assays increased from 97 to 128 during the period of study. Some other centers used ELISA for vWF activity rather than a vWF:RCo assay. The number of centers using such ELISAs for vWF activity decreased from 29 to 21 during the survey period.

Results of individual surveys are shown in Table 2 for users of aggregometry (i.e., use of platelet aggregometers or automated systems to monitor platelet agglutination), visual agglutination (i.e., use of manual test tube or slide-based techniques with visual observation to record agglutination), and vWF activity ELISA. The difference between results obtained by users of ELISA

vWF activity and vWF:RCo either by aggregation or by visual agglutination is shown in Fig. 1. There were no significant differences between the results obtained with each of these techniques. Results obtained by ELISA users were similar to those obtained by users of other techniques even when vWF:RCo was <10 IU/dL in type 2M vWD. The correlation coefficient between ELISA and aggregometry medians was 0.99. The mean CV for users of aggregation and visual agglutination was 51% and 49% compared with 41% for ELISA (not significant).

Three different aggregometry systems were used in 10 or more centers. There were no significant differences between mean results and CVs for these three

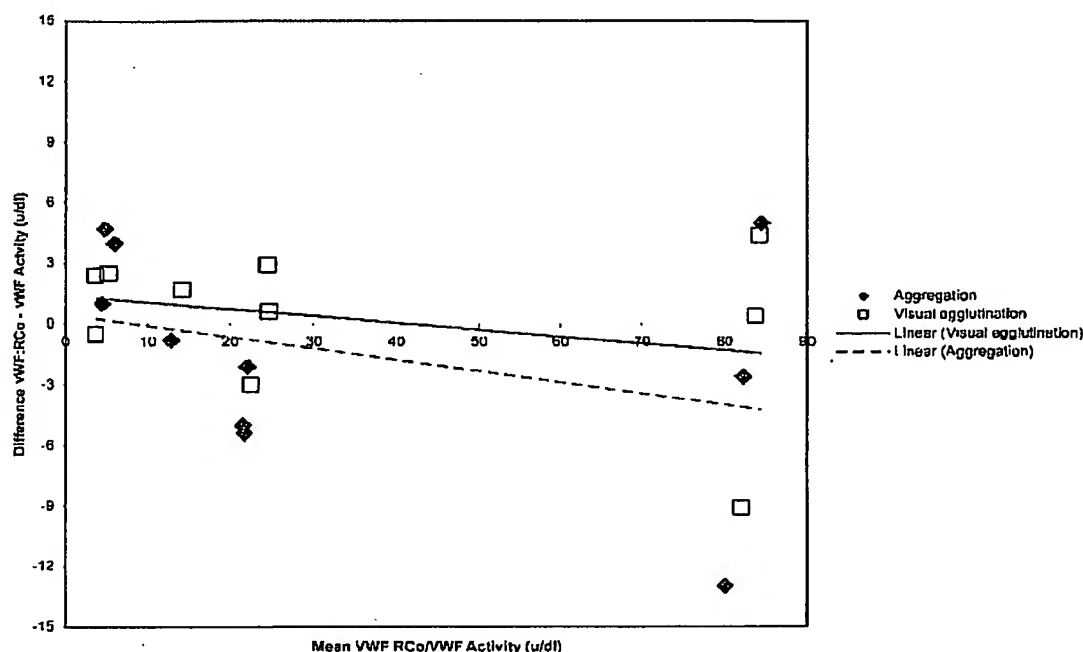
**Table 2 vWF:RCo and vWF Activity ELISA Results Grouped According to the Method Used in Participating Centers: UK NEQAS Participants' Data 2001–2005**

Date of Survey	Sample Type	Number of Users			Median (IU/dL)			CV (%)		
		Aggreg*	Visual Agg†	ELISA	Aggreg	Visual Agg	ELISA	Aggreg	Visual Agg	ELISA
January 2001	Type 2M	66	20	29	5.0	6.5	4.0	12.0	104	98.5
September 2001	Type 1	73	21	25	19.0	21.0	24	40.2	39.0	26.9
November 2001	Normal	73	20	30	73.6	77.5	86.6	23.8	22.5	15.7
May 2002	Type 2N	79	21	28	87.0	86.4	82.0	21.7	42.6	88.0
November 2002	Type 1	70	21	27	21.0	26.0	23.1	64.5	36.5	23.5
May 2003	Type 1	73	19	30	19.0	25.0	24.4	39.0	35.0	22.3
November 2003	Type 2M	73	19	18	7.2	4.9	2.5	69.2	57.2	84.9
May 2004	Type 2N	76	24	24	81.0	84	87.6	26.8	23.8	16.4
November 2004	Type 2M	77	22	25	8.0	3.5	4.0	61.1	84.1	76.6
May 2005	Type 1	80	16	21	12.5	15.0	13.3	48.5	43.0	40.2
Mean	—	74	20	26	33.3	35.0	35.2	61.5	49.1	41.3

\*Aggregation (i.e., using platelet aggregometers or automated systems to monitor platelet agglutination).

†Visual agglutination (i.e., manual test tube or slide-based techniques with visual observation to record agglutination).

vWF:RCo, von Willebrand factor:ristocetin cofactor activity; ELISA, enzyme-linked immunosorbent assay; UK NEQAS, United Kingdom National External Quality Assessment Scheme; CV, coefficient of variation.



**Figure 1** Bland-Altman plot of the difference between median von Willebrand factor:ristocetin cofactor activity (vWF:RCO) by aggregation or visual agglutination and vWF activity by enzyme-linked immunosorbent assay.

techniques (Table 3). There was a trend toward higher CVs at lower levels of vWF activity.

In May 2005, 13 centers used a latex immunoassay using a specific anti-vWF monoclonal antibody directed against the platelet-binding site of vWF (glycoprotein Ib receptor), adsorbed onto latex particles. This method had not been used in earlier surveys. The median result for users of this latex immunoassay was 18.0 IU/dL (CV, 27%) compared with 12.5 IU/dL (CV,

48.5%) for users of aggregometry, and 18.3 IU/dL (CV, 40.3%) for users of ELISA. This sample was from a subject with type 1 vWD.

#### vWF:Collagen Binding

Use of vWF:CB assays increased slowly during the period of study, with the number of users exceeding 10 in three surveys (beginning May 2004). For these

**Table 3** Results of vWF:RCO Obtained by Aggregometry Groups According to the Instrument Used: UK NEQAS Participants' Data 2001–2005

Date of Survey	Sample Type	Dade-Behring BCS (n = 19)*		Helena (n = 21)*		Biodata (n = 15)*	
		Median (IU/dL)	CV (%)	Median (IU/dL)	CV (%)	Median (IU/dL)	CV (%)
January 2001	Type 2M	15.0	80.2	2.0	—†	7.0	—†
September 2001	Type 1	17.2	45.2	18.7	40.0	19.0	33.9
November 2001	Normal	73.5	21.1	74.4	20.1	74.0	24.2
May 2002	Type 2	85.0	16.6	90.0	26.0	86.3	25.4
November 2002	Type 1	22.5	89.5	20.0	27.9	21.0	30.6
May 2003	Type 1	19.9	61.1	18.0	31.7	18.0	44.5
November 2003	Type 2M	9.6	40.9	5.0	81.1	10.5	—†
May 2004	Type 2N	85.0	17.6	72.8	27.6	88.0	32.6
November 2004	Type 2M	10.0	50.1	7.7	69.5	10.0	48.8
May 2005	Type 1	16.0	31.4	10.0	48.0	10.0	42.3
Mean	—	35.4	45.4	32.4	41.3	34.4	35.9

\*Average number of users of each instrument over the period of surveys.

†CV not calculated if less than 10 results were obtained by this technique.

vWF:RCO, von Willebrand factor:ristocetin cofactor activity; UK NEQAS, United Kingdom National External Quality Assessment Scheme; CV, coefficient of variation.

**Table 4 Ratios of vWF:RCo or vWF Activity ELISA to vWF:Ag: UK NEQAS Participant' Results in Surveys 2001-2005**

Date of Survey	Sample Type	Aggregometry (n = 63)*		Visual Agglutination (n = 17)*		ELISA (n = 25)*	
		Median	Range	Median	Range	Median	Range
January 2001	Type 2M	0.45	0.01-4.0	0.21	0.17-1.92	0.25	0.05-1.57
September 2001	Type 1	0.73	0.13-3.83	0.76	0.17-2.0	0.85	0.47-1.04
November 2001	Normal	0.74	0.29-1.24	0.80	0.47-1.32	0.87	0.60-1.30
May 2002	Type 2N	0.85	0.36-1.77	0.82	0.40-1.78	0.86	0.60-1.01
November 2002	Type 1	0.74	0.43-20.1	0.96	0.43-1.92	0.79	0.43-1.66
May 2003	Type 1	0.64	0.09-2.35	0.85	0.51-1.53	0.83	0.46-1.61
November 2003	Type 2M	0.80	0.02-4.81	0.51	0.03-1.63	0.30	0.07-1.47
May 2004	Type 2N	0.72	0.24-2.14	0.75	0.27-1.32	0.79	0.46-1.02
November 2004	Type 2M	0.73	0.01-1.86	0.43	0.05-2.0	0.27	0.01-1.13
May 2005	Type 1	0.75	0.05-1.75	0.88	0.28-2.23	0.72	0.47-1.59
Mean	—	0.71		0.70		0.65	

\*Average number of participants who returned vWF:RCo or vWF activity ELISA by each method who also returned a vWF:Ag result allowing calculation of a ratio.

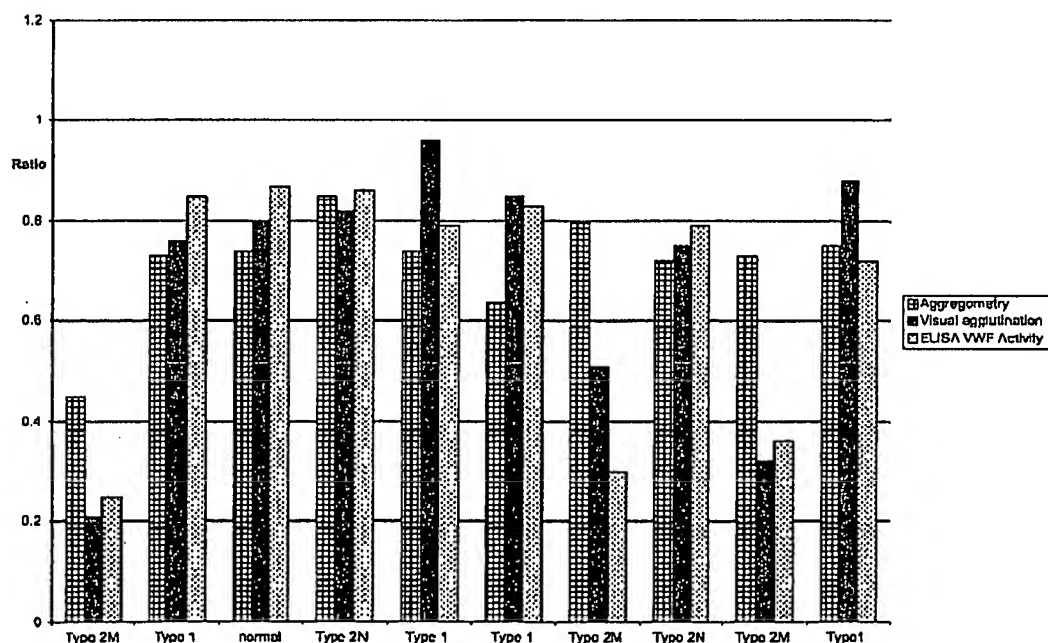
vWF:RCo, von Willebrand factor:ristocetin cofactor activity; ELISA, enzyme-linked immunosorbent assay; UK NEQAS, United Kingdom National External Quality Assessment Scheme; Ag, antigen.

three surveys, the median vWF:CB assay results were 97.0, 5.0, and 14.0 IU/dL, compared with 81.0, 8.0, and 12.5 IU/dL for users of aggregometry on samples from subjects with type 2N, type 2M, and type 1 vWD, respectively.

#### Ratios of vWF Activity to Antigen

A ratio of vWF activity to vWF:Ag was calculated for each participant returning both vWF activity and anti-

gen results in each individual survey. The median and range of ratios for each type of vWF:RCo or vWF activity assay are shown in Table 4 and Fig. 2. For every sample, a wide range of ratios was evident irrespective of the activity and antigen assay methods used. On all samples, some centers obtained ratios of 0.6 or lower, which would be compatible with type 2 vWD,<sup>2</sup> and other centers obtained ratios above 0.7, which would be compatible with type 1 vWD, and irrespective of whether the sample was actually from normal subjects,



**Figure 2** . Median ratio of von Willebrand factor (vWF) activity to vWF antigen in 10 samples calculated for users of vWF:ristocetin cofactor activity (RCo) by aggregometry, vWF:RCo by visual agglutination, or vWF activity by enzyme-linked immunosorbent assay (ELISA).

a patient with type 1 vWD or a patient with genetically confirmed type 2 vWD.

For samples from the patient with type 2M, the ratio of vWF activity to vWF:Ag was markedly lower for users of visual agglutination or ELISA when compared with the ratio derived from aggregometry results. At such low vWF levels, however, caution is required in the use of ratios.

For most surveys there were insufficient users of vWF:CB to draw conclusions about ratios of vWF:CB to vWF:Ag. However, in the three most recent surveys where vWF:CB results were returned by more than 10 centers, the median ratios and ranges of vWF:CB to vWF:Ag were as follows: May 2004 (type 2N), median ratio = 0.94 (range, 0.69 to 1.32); November 2004 (type 2M), median ratio = 0.45 (range, 0.22 to 1.0); and May 2005 (type 1 vWD), median = 0.80 (range, 0.47 to 1.15).

In January 2005, 27 of 70 centers (34%) who returned answers to a questionnaire indicated that they used a ratio of vWF activity to vWF:Ag in the diagnosis of vWD. Twenty-one of these used 0.7 as the cut-off between type 1 and type 2A/2B/2M vWD, with ratios of 0.4 to 0.65 used in the other six centers.

## DISCUSSION

Data from more than 180 centers during a 5-year period are reviewed. These include a substantial proportion (up to 45%) of centers from outside the United Kingdom. A series of samples from patients with type 1 vWD, type 2M vWD, and type 2N vWD as well as normal plasma were analyzed by these centers. During the period 2001 to 2005, there were several changes in the pattern of methods in use for determination of vWF levels and activity. In vWF:Ag testing, the use of ELISA technology decreased from 48% to 26% of users, with automated latex immunoturbidometric assays from two different manufacturers replacing ELISA in many centers. Immunoelectrophoresis practically disappeared from use over this period. The results obtained by the different techniques were in good agreement, suggesting that all are suitable at least for the types of samples included in the surveys. The CV of results in different centers was higher when vWF:Ag was below 20 IU/dL than for samples with higher levels of vWF:Ag. Although not significant, there was a trend to lower CVs for immunoturbidometric assays, possibly reflecting uniformity of reagent and instrument sources. The mean CV was 15 to 20% for different methods, which is similar to that reported for vWF:Ag results in International Haemophilia Training Centres in the period 1994 to 1997,<sup>8</sup> and similar to those obtained by participants in a recent report from the Australian EQA program.<sup>15</sup> A small number of centers are now using fluorescence-based ELFA assays for vWF:Ag, and to date the results have been in agreement with those obtained by users of other

methods, with CVs less than half those by other antigen assays.

Probably the most important single laboratory test in the diagnosis of vWD has historically been the vWF:RCO assay. In the past, there has been poor agreement between results in different centers using the same technique, as indicated by high CVs. We have previously reported CVs up to 64% even among an expert group of International Haemophilia Training Centres<sup>8</sup> for a sample with vWF:RCo of 11 IU/dL. There has been no improvement during 2001 to 2005, when average CVs of 40% to 50% were noted for users of aggregometry and visual agglutination. Even for users of the same instrument for aggregometry, the between-center CVs were 35% to 45%. This high degree of imprecision in vWF:RCo contributed to the establishment of ELISA techniques for vWF activity using antibodies directed against functional epitopes of vWF,<sup>9</sup> with the potential for improved precision that ELISA might allow. However, the average CV for ELISA users who analyzed the 10 samples in these exercises was 41%. An early commercial ELISA was found to be in poor agreement with vWF:RCo assays in type 2A vWD,<sup>10</sup> type 2B vWD,<sup>11</sup> and type 2M vWD<sup>11,12</sup> and this may have contributed to recommendations that a platelet-based assay for vWF:RCo should be used in preference to such ELISA assays for vWF activity.<sup>1</sup> Other ELISA vWF activity assays have also been described.<sup>13</sup> Most of the centers using ELISA for vWF activity used the same commercial kit, which was modified in 1999.<sup>14</sup> For the samples circulated from a subject with type 2M vWD, the ELISA showed good agreement with vWF:RCo assays performed by aggregometry or visual agglutination, even when the median vWF:RCo was ~5 IU/dL. More data on type 2 vWD would help to confirm that this revised assay is suitable for characterization of type 2 vWD.<sup>4</sup>

vWF:CB has been recommended as an additional test rather than a replacement of vWF:RCo,<sup>1,3,15</sup> and was consistently used by ~50% of centers that participated in Royal College of Pathologists of Australasia Quality Assurance Programmes between 1998 and 2004.<sup>16</sup> Uptake of the vWF:CB has been much lower among participants in the United Kingdom program, with less than 10% using this assay during the period reviewed; this result prevents meaningful conclusions about the performance of this assay in this setting.

The ratio of vWF:RCo to vWF:Ag can be a useful diagnostic tool because it is markedly decreased in type 2A and reduced in type 2B and type 2M vWD<sup>2</sup> compared with type 1 vWD. In one study,<sup>11</sup> the lower limit of ratio in normal subjects was 0.7; ratios were below 0.7 for patients with type 2B or type 2M and below 0.3 for type 2A vWD. More recently, Federici et al<sup>17</sup> used 0.6 as the lower limit of normal ratios based on the normal practice in five collaborating expert centers. In the current study, we calculated ratios of



vWF:RCo or vWF activity to vWF:Ag for centers returning both results. Ratios in samples from patients with type 2M vWD were markedly lower for users of ELISA vWF activity or visual agglutination when compared with users of aggregometry. However, these samples had levels of vWF activity below 10 IU/dL, making the use of ratios imprecise. Overall, our data suggest that the ratio may differ according to the technique in use and we therefore recommend that centers should determine lower limits for ratios using the methods in local use. For all of the vWF:RCo and activity methods and for all of the samples distributed (including a normal sample), some centers obtained ratios compatible with type 1 vWD, whereas others reported results compatible with type 2 vWD.

Overall, our surveys indicate that laboratory testing for vWF remains problematic. Results are imprecise by most of the techniques currently used. It remains to be seen whether some newer techniques will offer consistently improved precision.

#### ACKNOWLEDGMENTS

The authors thank Dr. Anne Goodeve and Dr. Mandy Nesbitt for genetic analysis of type 2 vWD and Margaret Marshall for secretarial assistance.

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# DADE BEHRING

## BC von Willebrand Reagent

for the determination of ristocetin cofactor activity

### Intended Use and Application

In vitro diagnostic reagent for determination of the ristocetin cofactor activity of von Willebrand factor in human plasma using the platelet agglutination method.

### Summary and Explanation

Von Willebrand disease is a common autosomal human bleeding disorder caused by the defective synthesis or function of the multimeric von Willebrand Factor. Von Willebrand Factor is present in the plasma in the form of a complex with F VIII. Von Willebrand's disease is the most common congenital bleeding disorder. It has an autosomal dominant mode of inheritance. There are at least six different forms: I, IIA, IIB, III, hemophilia-type, and platelet-type. The variants are differentiated on the basis of a panel of laboratory tests that include the bleeding time, platelet count, partial thromboplastin time, von Willebrand activity and antigen, Factor VIII levels, ristocetin cofactor activity, and von Willebrand multimeric composition<sup>1</sup>.

The BC von Willebrand Reagent is used for the determination of von Willebrand factor ristocetin cofactor activity to aid in the diagnosis of von Willebrand disease and of diseases with changes in the ristocetin cofactor activity, such as vascular endothelial lesions, inflammations, and preoperative screening of bleeding tendency.

### Principle of the Method

The Dade Behring Marburg GmbH BC von Willebrand assay measures ristocetin cofactor activity as follows: von Willebrand factor (ristocetin cofactor) from the sample causes agglutination of stabilized platelets (provided by the von Willebrand Reagent) in the presence of ristocetin. The resulting agglutination decreases the turbidity of the reaction suspension. A coagulation instrument measures the change in absorbance and automatically determines the sample's ristocetin cofactor activity in % of the norm.

### Reagents

#### Materials provided

Code No. OUBD: 5 x for 4 ml of BC von Willebrand Reagent

#### Composition

The BC von Willebrand Reagent contains stabilized platelets, ristocetin and EDTA in lyophilized form.

Preservative: sodium azide (max. 0.5 g/l)

#### Warnings and Precautions

1. For in vitro diagnostic use only.
2. Reagents containing sodium azide must be handled with due caution: Do not ingest or allow to contact skin or mucous membranes! Sodium azide can form explosive azides when contacting heavy metals such as copper or lead!
3. Each individual blood donation for use in manufacture of BC von Willebrand Reagent is tested for hepatitis B surface antigen, anti-HCV, anti-HIV1 and anti-HIV2. Only donations with negative findings are used for manufacture. Nevertheless, since absence of infectious agents cannot be proven, all materials obtained from human blood should always be handled with due care, observing the precautions recommended for biohazardous material<sup>2</sup>.

#### Preparation of the Reagent

Resuspend the reagent in the labelled quantity of distilled water at +15 to +25°C by shaking (e.g. on an automatic shaker). The reagent is immediately ready for use.

#### Storage and Stability

Store the reagent unopened at +2 to +8°C and use by the expiry date given on the label.

Stability after reconstitution:

at +2 to +8°C	2 days
at +15°C (opened container)	8 hours

#### Materials Required But Not Provided

Standard Human Plasma, Code No. ORKL

Control Plasma N, Code No. ORKE

Control Plasma P, Code No. OUPZ

### Specimen Collection and Preparation

To separate the plasma, mix 1 part sodium citrate solution (0.11 mol/l) with 9 parts venous blood, taking care to avoid the formation of foam. Centrifuge immediately at no less than 1500 x g for at least 10 min, remove the supernatant plasma, and keep at +15 to +25°C until required in the assay, or store deep-frozen.

Stability of the specimen:

6 hours	at +15 to +25°C
1 month	at -20 to -30°C

### Method

#### Procedure

Procedure on the Behring Coagulation System (BCS) and the Behring Coagulation Timer (BCT): A special instruction manual for the BCS and the BCT is available.

On the BCT, ensure that the reagent vial is provided with a magnetic stirrer and placed into reagent well 1 or 3. If an analyzer without a stirrer well is used, ensure that the reagent is resuspended by shaking for not more than 30 min.

#### Internal Quality Control

Normal Range: Use Control Plasma N

Pathological Range: Use Control Plasma P

Suitable controls must be run for each vial of the reagent (e.g. Control Plasma N, Control Plasma P). If the control is repeatedly outside the assigned range, recalibrate. The measurements are to be run in duplicate. The analytical value obtained must be within the assigned range given for the control in the lot-dependent table of values.

#### Results

The ristocetin cofactor activity is expressed in % of the norm.

#### Limitations and Interferences

In some cases of type IIB von Willebrand's disease, normal or borderline ristocetin cofactor activities were reported<sup>1</sup>. Treatment of patients with FVIII: c concentrates or DDAVP (1-deamino-[8-D-arginine]-vasopressin) may raise ristocetin cofactor activity levels in patient plasma<sup>1</sup>.

### Expected Values

Each laboratory should establish its own reference range. Values below the established reference range can be considered to be an indication of von Willebrand's disease.

A dependence on blood group (ABO type) and age of von Willebrand Factor levels has been reported<sup>3</sup>.

### Specific Performance Characteristics

#### Precision

Over a five day period, precision studies (one run per day in replicates of eight) were performed by assaying normal and pathological control plasmas. For normal control plasmas (n = 80), the within-run precision ranged from 8.0 to 9.6%, while the total precision ranged from 8.0 to 10.3%. For pathological control plasma (n = 80), the within-run precision ranged from 6.1 to 16.2%, while the total precision ranged from 7.6 to 16.9%.

#### Method Comparison

A method comparison study was performed with the BC von Willebrand Reagent and another commercially available assay. A total of 70 patient plasma samples (35 normal and 35 abnormal) were tested by both methods. Regression analysis determined a correlation coefficient of 0.94, a y-intercept of -4.4, and a slope of 0.97.

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Manufacturer: Dade Behring Marburg GmbH  
Emil-von-Behring-Str. 76  
D-35041 Marburg/Germany

USA Distributor: Dade Behring Inc.  
Newark, DE 19714 U.S.A.